Improvements in biological hydrogen production by microbial fermentations

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ABSTRACT

Hydrogen is being touted as an ideal future green fuel. Various biological means for the renewable production of hydrogen are being investigated. Biohydrogen production by microbial fermentation is a process that could use readily available wastes, as well as presently unutilized bioresources. In order to make this process practical, yields must be increased. Recent results show that conversion efficiencies can be improved through nutrient limitation, and genetic modification to increase hydrogenase expression and eliminate competing pathways. Results show that dilution rate is an important factor controlling the overall efficiency of an hydrogen fermentation, with a trade-off between increased production and increased substrate conversion. Biodiesel-derived glycerol is currently produced in large amounts, and its production will dramatically increase in the future. Here we show that photofermentation can be used to stoichiometrically convert glycerol to hydrogen.

Keywords: biofuels, biohydrogen, fermentation, hydrogen yields, glycerol conversion

1 INTRODUCTION

Soaring crude oil costs, decreasing quantities of finite petroleum reserves, and concern for abatement of greenhouse gas emissions leading to climate change are driving a search for green technologies for producing alternative fuels. Using, either directly or indirectly, solar energy captured biologically to generate a biofuel is certainly attractive. Many possible biologically derived energy carriers have been proposed. Three different carriers have achieved major attention; biodiesel, bioethanol, and biohydrogen. In order for biofuels to make a significant impact on the reduction of use of fossil fuels and greenhouse gas emissions, they will need to be made on an enormous scale. Production of biofuels, both biodiesel and bioethanol, is already at a large magnitude and is projected to greatly increase in the future. Thus, concerns about the environmental and social impacts of biofuel production itself are growing.

Hydrogen is poised to become an important future energy carrier. Hydrogen may ultimately be the best alternative, renewable energy carrier. In many ways hydrogen is a superior future energy carrier. For example, hydrogen has many potential advantages over bioethanol. Its production should be less energy intensive; being a gas it is easily separated from the fermentation broth thus avoiding the distillation required to recover ethanol. Hydrogen could possibly be used in the future to power fuel cell vehicles at an energy conversion yield approximately twice that of combusting ethanol in an ICE. Use of hydrogen as a transportation fuel, emitting water vapour, would be less polluting than the use of ethanol since ethanol combustion leads to the formation of ozone and the emission of known carcinogens, such as acetaldehyde.

Renewable hydrogen production is key to making it a truly sustainable replacement for fossil fuels, and for realizing its full potential in reducing greenhouse gas emissions. However, the biological production of hydrogen, although under active development [1, 2] is still at the scale of basic laboratory research. We have been investigating biological hydrogen production by microbial fermentation, a process that could use untapped bioresources and already available waste streams. Although these could also in principle be converted by fermentation to ethanol, an additional advantage of the fermentative production of hydrogen is that the carbon dioxide derived from the substrate is produced at the source, potentially allowing its capture and sequestration, rather than being released during combustion, as is the case with bioethanol. However, in order to make this process practical, yields must be increased

Novel problems are being engendered by biofuel production.. Glycerol is generated during biodiesel fabrication, and due to the presently large, and projected enormous, scale of biodiesel production, its further use or disposal has become problematic. The projected future magnitude of biodiesel-derived glycerol production mandates that it be converted into a bulk chemical that can be used as a fuel.

2 MATERIALS AND METHODS

2.1 Fermentative hydrogen production

The culture conditions and *Escherchia coli* strains used for fermentative hydrogen production studies have been previously described in detail [3,4]. Briefly, media were formulated as modified M9 [5] containing glucose. Batch cultures were grown anaerobically (37 ^{o}C) in 9.7 vials sealed with butyl rubber stoppers. Continuous cultures were carried out using automated chemostats (37 ^{o}C) maintained anaerobic by sparging. The *Escherichia coli* strains used were BW535 and its hyd1- hyd2- derivative,

Clean Technology 2008, www.ct-si.org, ISBN 978-1-4200-8502-0

JW135, LJT135 (JW135 *ldhA*::Tn*10*), FJT135 (JW135/pWS165 (FhlA-C) and DJT135 (JW135 *ldhA*::Tn*10*/pWS165).

2.2 Photofermentation of glycerol

Photofermentation of glycerol used cultures of the non-sulfur photosynthetic bacterium, *Rhodopseudomonas palustris* CGA009. Cultures were grown on RCV medium [6] containing 10mM glycerol, 0.05% yeast extract, and 0.05% sodium bicarbonate with the indicated concentrations of glutamate as nitrogen source. Cultures were illuminated with a bank of 50 W spotlights, placed at a distance of 25 cm from the cultures contained in 125 ml serum bottles which were sealed with rubber stoppers and placed in a thermostated (30° C) water bath. The headspace of the culture flasks was connected by FEP 890 tubing to inverted glass graduated cylinders which were used to measure gas production by displacement.

2.3 Measurement of hydrogen

Hydrogen was measured by injecting an aliquot of the headspace gas into a Shimadzu GC8 chromatograph equipped with a thermal conductivity detector. The 2 meter column contained molecular sieve 5A with argon as the carrier gas.

3 RESULTS AND DISCUSSION

3.1 Fermentative production of hydrogen

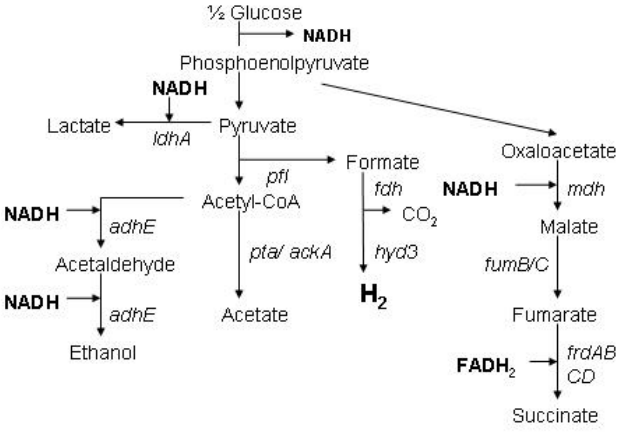

Figure 1 Mixed-acid fermentation

Possible fermentation products are shown along with the relevant genes encoding the enzymes involved in metabolite interconversions. Reactions generating or consuming NADH are indicated. H_2 is produced by the *fhl* pathway, specifically Hyd3. Hyd 1 and 2 (not shown), if present, consume H_2. Adapted from [4].

E. coli carries out a mixed acid fermentation (Figure 1) with a variety of possible products. In principle product distribution is governed both by the oxidation/reduction state of the substrate, with more reduced substrates producing a larger proportion of reduced products, e.g. ethanol, and by environmental factors, especially the pH. At lower pHs, lactate production is increased, and the *fhl* pathway is induced, leading to decomposition of formate to H_2 and CO_2. As we have previously shown in both batch [3] and continuous cultures [4], elimination of competing pathways; hydrogen consumption by Hyd1 and Hyd2, or lactate production by lactate dehydrogenase (*ldhA*), increases net hydrogen production. As well, increasing Hyd3 activity by expressing a constitutive allele (FhlA-C) of the activator for the *fhl* pathway, FhlA, can also increase hydrogen production.

The greatest effect was seen when hydrogen consumption activity was eliminated by introducing deletions in both *hyd1* and *hyd2*. Thus, JW135, an isogenic derivative of BW135 carrying △*hya-Km*, △*hyb-Km*, produces 37% more hydrogen than the parental strain [3].

Table 1. Effects of various mutations on hydrogen yields with batch cultures with limiting glucose

Strain	*Genotype*	*Yield ($molH_2$/mol glucose) 0.03% glucose*	*Yield ($molH_2$/mol glucose) 0.05% glucose*
JW135	△*hya-Km* △*hyb-Km*	1.24	0.71
LJT135	*ldhA*::Tn*10*	1.49	1.12
FJT135	pWS165 (FhlA-C)	1.24	1.22
DJT135	*ldhA*::Tn*10*, pWS165 (FhlA-C)	1.98	1.83

In addition, introduction of a mutation in lactate dehydrogenase, which diverts electron flow away from hydrogen, or increasing formate degradation by constitutively expressing *fhl* can also increase net H_2. With the hyd1-, hyd2- strain JW135, this is most obvious at limiting glucose concentrations, as shown in Table 1. Under these conditions, hydrogen yields were significantly increased (20% at 0.03% glucose, 69% at 0.05% glucose) by eliminating lactate dehydrogenase activity (strain LJT135). Yields were also increased, at least at 0.05% glucose, by expression of FhlA-C (strain FJT135). Finally, a strain carrying both these alterations, strain DJT135, gave the highest yields, approaching the theoretical maximum of 2 at these limiting glucose concentrations (Table 1).

The effects of some other limiting nutrients on hydrogen yields have also been investigated [3]. Sulfur (sulfate) and

phosphate limitation do not appear effective in controlling H_2 yields, with a maximum of 1 mol H_2 / mol glucose being attained with these limiting nutrients. On the other hand, nitrogen limitation, like glucose limitation, also appears to be effective in manipulating hydrogen yields with nearly stoichiometric yields (2 mol H_2/ mol glucose) being obtained at low (~1 mM) nitrogen concentrations.

The mechanism(s) by which nitrogen or glucose, as limiting nutrients, affect hydrogen yields is unclear. Limitation in nitrogen, by affecting bacterial growth, may increase substrate conversion to hydrogen at the expense of biosynthesis of new cellular material at low cell densities.
It is less obvious why limiting the cells for glucose leads to what appears to be more efficient substrate utilization. Possibly carbon flow to secondary pathways of metabolism, for example glycogen synthesis, is restricted at low glucose concentrations thereby shunting the majority of the substrate into hydrogen production.

Studies carried out with continuous cultures have corroborated the batch studies [4]. Hydrogen yields for strain DJT135 were found to be higher than those of JW135 at all dilution rates tested. In general, hydrogen yields (mol H_2/mol glucose) appeared to be affected by dilution rate, with yields decreasing with increasing dilution rate for all nutrient limitations (sulfur, phosphate, nitrogen, and glucose) examined. Thus hydraulic retention time can be an important factor affecting the efficiency of hydrogen fermentations. On the other hand, hydrogen production increased (up to a point) with increasing dilution rate. Thus there appears to be an inverse relationship between these two parameters. This could be important from an operational standpoint with a trade off between yields of substrate conversion and overall productivity.

The maximal volumetric hydrogen production rates which varied from 1.3 to 5.3 mmol H_2/l/h, are similar to what has been found in some other studies of a variety of bacteria using continuous cultures limited for glucose.

3.2 Photofermentation of glycerol

The major drawback to the biological production of hydrogen from sugar substrates has been the low yields usually obtained. Analysis of the metabolic pathways normally used during fermentation predicts a maximum theoretical yield of 4 H_2/mol GE (glucose equivalent), and in practice, yields are usually at least 25% below this [2]. Since $12H_2$ can potentially be derived from glucose, fermentation thus functions at best at a 33% substrate conversion efficiency. What are the prospects for making hydrogen from glycerol? In a normal anaerobic fermentation, glycerol enters the microbial cell and is metabolized to pyruvate.

Pyruvate can be further catabolized by two different enzyme systems. However, in both cases a theoretical maximum of $1H_2$ is produced per molecule of glycerol. Indeed, hydrogen production from glycerol waste has been previously reported with yields of 0.53 mol/mol [7] up to 0.77 mol/mol [8]. This is the typical problem encountered in biohydrogen production through fermentation; many microbial dark anaerobic fermentations are capable of producing hydrogen from a variety of substrates. However, none can catalyze the complete extraction of all the hydrogen potentially present in the substrate (reviewed in [2]). The end result of pyruvate degradation is to produce acetyl-CoA in addition to a molecule from which hydrogen can be derived. As there is no known pathway for the dark, anaerobic conversion of acetyl-CoA to hydrogen, this fraction of the initial substrate is "lost" to hydrogen production.

These barriers can be overcome by photosynthetic bacteria which are capable, under the proper conditions, of using captured solar energy to drive the complete conversion of substrate to hydrogen. This process has been called photofermentation. Under these conditions, nitrogenase, whose physiological function is to reduce N_2 to NH_3, reduces protons to hydrogen. Hydrogen production from carbon containing substrates can only be demonstrated in media which is poor in fixed nitrogen. Complete conversion of the protons in glycerol to hydrogen, as is possible with this process, would yield eight moles of hydrogen per mole of glycerol, or eight times what is available from a conventional dark fermentation. Here we have examined the photofermentation of glycerol by *Rhodopseudomonas palustris* CGA009 using a minimal medium (RCV) with a nitrogen source, glutamate, that allows nitrogenase synthesis and activity.

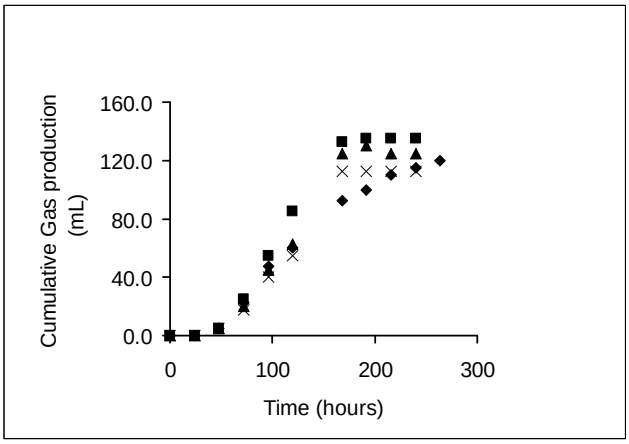

Figure 2. Photofermentative hydrogen production from glycerol by *Rhodopseudomonas palustris*

Cultures were grown photosynthetically on minimal RCV medium containing 10 mM glycerol as described in Materials and Methods and different concentrations of glutamate as nitrogen source: ◆, 0 mM; ■, 2 mM; ▲, 4 mM; ✕, 8 mM

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As shown in Figure 2, this non-sulfur photosynthetic bacterium was capable of light-driven hydrogen production from glycerol at various initial glutamate concentrations. All cultures showed a significant lag, ~50 hours, before hydrogen production commenced. The different cultures showed similar kinetics of hydrogen production and hydrogen production in all cultures ceased by ~200 hours.

Table 2. Yields of H_2 from glycerol at different glutamate concentrations

[Glutamine] (mM)	*Yield (Moles H_2/mole glycerol)*
0	5.2
2	6.4
4	5.9
8	3.8

We examined the stoichiometry of glycerol conversion by measuring the amount of gas evolved and its hydrogen content as well as chemically assaying residual glycerol concentrations. The results are shown in Table 2. Lower initial glutamate concentrations appeared to give higher conversion yields, perhaps because the lower total nitrogen content supported less growth. This has the dual effect of diverting less substrate into cellular material while at the same time, lower cell densities might increase energy efficiency by reducing self-shading. At 2 mM glutamate, 6.4 moles of H_2 were obtained per mole of glycerol consumed, this is 80% of the maximum stoichiometry of eight theoretically obtainable.

4 CONCLUSIONS

Here some strategies have been presented and shown to be capable of increasing hydrogen production by an organism, *E. coli*, carrying out a mixed-acid fermentation. Genetic manipulation; inactivation of the uptake hydrogenases, mutation of the lactate dehydrogenase gene (*ldhA*), and increased expression of the *fhl* pathway through expression of FhlA-C, all increase hydrogen yields. Batch or continuous cultures of suitably mutated strains grown with limiting glucose or nitrogen show yields (mol H_2 / mol glucose) approaching the predicted metabolic maximum of 2. Studies with continuous cultures show that there is potentially a trade-off between hydrogen production rates and yields. Nonetheless, the maximum yields obtained in numerous studies are well below the theoretical 12 H_2 per glucose. The challenge to making the dark fermentative production of hydrogen from carbohydrate containing materials a practical process is to develop processes that approach this maximum.

Many studies have previously shown that photosynthetic bacteria are capable of the stoichiometric photodissimilation of organic acids to hydrogen. This is the first time that a photosynthetic bacterium has been shown to be capable in principle of the nearly stoichiometric photofermentative conversion of glycerol to hydrogen. However, several factors, including the established low light conversion efficiencies of these organisms, and the potentially high cost of transparent, hydrogen-impermeable, photobioreactors, present very significant hurdles for the development of a practical process.

ACKNOWELDGEMENTS

This research was supported by grants from the Natural Sciences and Engineering Research Council of Canada under the Discovery Grants and Strategic Research Grants programs. G. Sabourin-Provost was supported by a fellowship from FQNRT.

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