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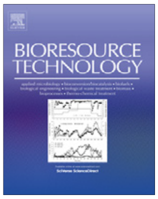

Photofermentation of crude glycerol from biodiesel using *Rhodopseudomonas palustris*: Comparison with organic acids and the identification of inhibitory compounds

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HIGHLIGHTS

- Growth and H_2 production by *R. palustris* on various carbon sources investigated.
- Saponified fatty acids identified as the inhibitory component of crude glycerol.
- Investigations into inhibition by glycerol, alcohols and salts conducted.
- Normal growth and H_2 production on crude glycerol after treatment shown.
- Significant results in photofermenting crude glycerol to high purity H_2.

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ABSTRACT

The growth of the biodiesel industry, and its concurrent production of glycerol, has lowered the price of glycerol 20-fold. While many options for using this glycerol have been proposed, the size of the waste stream means that generation of fuels is likely to be the only viable route. One such fuel is hydrogen, production of which can be achieved biologically. The photofermentation of glycerol to hydrogen using *Rhodopseudomonas palustris* was investigated by exploring the growth rate, hydrogen production rate and hydrogen yield. *R. palustris* grows on glycerol at a rate of $0.074\ h^{-1}$, and photoferments glycerol into 97 mol% hydrogen at a conversion efficiency nearing 90% of the 7 mol H_2 theoretical maximum at a rate of 34 ml $H_2/g_{dw}/h$. Some inhibition of growth by crude glycerol was seen. This was determined to be caused by saponified fatty acids, removal of which yielded a treated crude glycerol which showed no inhibition.

1. Introduction

The international impacts and repercussions of global climate change resulting from greenhouse gas emissions are increasingly a concern for policy makers. The political pressure this has exerted, as well as the market pressures of increasing crude oil price and decreasing oil reserves, has led to a surge in research into the production of alternative fuels, which, on substitution for fossil fuels, have the potential to reduce the rate of anthropogenic emission of CO_2. Many alternative fuels are made from biological feedstocks, and so are called biofuels. One such fuel, biodiesel, has seen a large and continuing increase in production (Bozbas, 2008). Biodiesel is manufactured from plant or animal oils by trans-esterification of triacyl glycerides with an alcohol, usually methanol or ethanol, to make biodiesel from the fatty acid portion, leaving glycerol as a by-product. The inherent production of glycerol from the biodiesel process is substantial: about 0.1 m^3 of glycerol is produced per 1 m^3 of biodiesel (Thompson and He, 2006).

Owing to the increase in glycerol production associated with an expansion in the manufacture of biodiesel, the price of glycerol has plummeted from about $2/kg in 2002 to about $0.10/kg currently, so that many manufacturers of biodiesel now merely discard it (Johnson and Taconi, 2009). This has opened up an opportunity for finding a suitable use for this waste glycerol.

One option is to produce hydrogen from the glycerol using a photosynthetic bacterium. This has a number of advantages. Firstly, biological production of hydrogen circumvents the problem of producing a dilute aqueous product stream, likely to be obtained

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if the glycerol were converted to liquid-phase products in a biological process. Hydrogen is relatively insoluble in water and so partitions itself almost entirely into the gas phase, dramatically reducing purification costs compared with the production of a soluble biochemical (Ghosh et al., 2012). Secondly, hydrogen is a useful commodity chemical of considerable interest as an energy carrier, particularly for use in fuel cells (Crabtree et al., 2004).

For the biological production of hydrogen from glycerol two options are available: fermentation or photofermentation. There is a significant body of work on the fermentation of substrates, including glycerol, in the dark to produce methane or hydrogen, using species such as *Halanaerobium saccharolyticum, Klebsiella pneumoniae, Enterobacter aerogenes*, genetically modified *Escherichia coli* and mixed cultures (Kivistö et al., 2011; Wu et al., 2011; Jitrwung and Yargeau, 2011; Yazdani and Gonzalez, 2007; Selembo et al., 2009). Hallenbeck's (2009) review suggests that conversion efficiencies in dark fermentation only reach 33%, and incomplete oxidation of the carbon sources results in the production of organic acids as waste products.

On the other hand, photofermentation utilises a light source as part of the metabolic production of hydrogen. Under photofermentative conditions the purple bacterium *R. palustris* can convert an organic carbon source into hydrogen using the enzymes nitrogenase and hydrogenase, which catalyse the oxidation according to the general equation:

$$C_xH_yO_z + (2x - z)H_2O \rightarrow (y/2 + 2x - z)H_2 + xCO_2$$

For the most part, growth and hydrogen production from *R. palustris* have been reported using as substrates organic acids, such as acetic, lactic, succinic, butyric or malic acids (Vincenzini, 1982; Carlozzi and Sacchi, 2001; Carlozzi and Lambardi, 2009; Chen et al., 2007, 2008). However, Sabourin-Provost and Hallenbeck (2009) and Ghosh et al. (2012) have shown that photofermentation of glycerol is possible, and that about 6 mol H_2 per mol glycerol, or 86% of maximum stoichiometric conversion, can be achieved, with there being some inhibition of *R. palustris* by crude glycerol.

The conversion efficiency of the dark fermentation process, using *Enterobacter* species for example, can be greatly improved by the addition of a second, photofermentative, step using *R. palustris*. As noted above, the by-products of incomplete oxidation of substrates in dark fermentation are organic acids, which can be converted, in turn, to hydrogen by photofermentation (Basak and Das, 2006). Several authors have therefore suggested the process route of using a photofermentative operation after dark fermentation to increase the net conversion of the substrate to hydrogen (Sarma et al., 2012; Hallenbeck et al., 2012; Nath et al., 2008; Lo et al., 2011; Lee et al., 2002). Whilst such a two-stage process is an improvement on a single dark fermentation in terms of conversion, it presents additional process complexity at the industrial scale, especially in situations where the organism used in the dark fermentation is inhibited by the impurities in crude glycerol, which include methanol, soap and sodium chloride (Sarma et al., 2012; Ito et al., 2005). Thus, it might prove more practicable to enact a single stage operation, using just photofermentation.

The current understanding of the conversion of crude glycerol into hydrogen by photofermentation, which has been expounded in a number of papers (*e.g.* Sabourin-Provost and Hallenbeck, 2009; Ghosh et al., 2012) is limited in several key respects. There has been little work done on a direct comparison of growth and hydrogen production from many likely carbon sources, including glycerol and crude glycerol, under the same conditions, nor have the inhibitory components in crude glycerol been identified or a method for their removal demonstrated. This study examines these issues, adding to current understanding of the conversion of glycerol in a single photofermentation step.

2. Materials and methods

2.1. Bacterial strains and growth conditions

The purple non-sulphur bacterium *R. palustris* (NCIMB 11774) was purchased from NCIMB and used for all experiments. The growth medium consisted of: (per litre) 0.2 g yeast extract, 1.7 g K_2HPO_4, 1.7 g KH_2PO_4, 0.2 g $MgSO_4{\cdot}7H_2O$, 0.05 g $CaCl_2{\cdot}2H_2O$, 0.4 g NaCl, 0.15 g $Na_2S_2O_3$, 0.005 g ferric citrate, 0.002 g para-aminobenzoic acid and 1 ml of trace element solution containing: (per litre) 70 mg $ZnCl_2$, 100 mg $MnCl_2{\cdot}4H_2O$, 60 mg H_3BO_3, 200 mg $CoCl_2{\cdot}6H_2O$, 20 mg $CuCl_2{\cdot}2H_2O$, 20 mg $NiCl_2{\cdot}6H_2O$ and 40 mg $NaMoO_4{\cdot}2H_2O$. To the growth medium was also added 1 ml of vitamin solution containing: (per litre) 1.2 g thiamine HCl and 0.01 g cyanocobalamin, filtered sterile. The pH of the growth medium was adjusted using HCl and NaOH to pH 7.4 ± 0.1.

In some of the experiments reported here, growth on organic acids was studied. Stock solutions of the acids were made up and adjusted to the operating pH of 7.4 ± 0.1, using NaOH, before addition to the growth medium. To prepare crude glycerol for use in media it was neutralised to pH 7.4 with 10 M HCl. Treated crude glycerol (TCG) was prepared by adding 25 g of solid $CaCl_2$ per 1 l neutralised crude glycerol in a beaker stirred by magnetic stirrer and flea at room temperature. The calcium precipitated most of the saponified fatty acids, which separated readily. The solid calcium soap was then removed by filtration through Whatman GF/A glass microfibre filter paper. One experimental series utilised one of 100% methanol, 100% ethanol, 2 M NaCl, 2 M KCl or 20 g/l sodium oleate when examining inhibition. These experiments were conducted by setting up growth flasks containing 50 mM glycerol and 10 mM glutamate and adding the appropriate volume of alcohol, salt or soap to make up the desired final concentration.

For continuous preculture the above growth medium was supplemented with pure glycerol and sodium glutamate to give final concentrations of, respectively, 100 mM and 20 mM. Cultures were grown anaerobically under an argon atmosphere, in 100 ml conical flasks, which were sealed and agitated by magnetic stirrer and flea and illuminated by fluorescent and incandescent light bulbs (Sylvania, standard F15W/35-535) giving about 50–60 μmol photons/m^2/s (Skye Instruments Quantum Sensor). For an inoculum, 1 ml of preculture in late exponential phase (OD $\sim$ 1) was used.

Periodically, the optical density of the cell suspension was determined using a spectrophotometer (Thermospectronic UV1). The dry cell mass was correlated with the optical density at 660 nm using the following equation, derived by filtering 20 ml aliquots of bacteria at various optical densities through 0.22 μm weighed filter paper to determine biomass: Dry Cell Mass (g/l) = 0.4 × absorbance at 660 nm, with a standard deviation of ±0.05 g/l.

Each experiment was repeated between 3 and 5 times to determine standard deviation, which is displayed as error bars on the figures.

2.2. Experimental method

Cultures were grown in either 100 ml conical flasks or 500 ml bottles. The flasks were used for metabolic studies on substrate use and the bottles for measurements of the production of hydrogen.

In order to create an oxygen- and nitrogen-free atmosphere, the cultures were sparged with argon for 10 min and the flask sealed