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Glycerol and mixture of carbon sources conversion to hydrogen by *Clostridium beijerinckii* DSM791 and effects of various heavy metals on hydrogenase activity

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ABSTRACT

Hydrogen is a carbon-neutral energy feedstock which is produced during fermentation of various carbon sources. The genomes of clostridia encode mainly [Fe-Fe]-hydrogenases. *Clostridium beijerinckii* DSM791 performed anaerobic fermentation of glycerol in batch culture at pH 7.5 and pH 5.5 and produced H_2. At pH 7.5, the glycerol consumption rate was 3.7 g/g cell mass/h, which was higher than that at pH 5.5. H_2 production reached 5 mmol/h/g cell mass at pH 7.5. The specific hydrogenase activity was ~1.4 fold higher if cells were grown on glycerol compared to cells grown on glucose. Single (Fe^{2+}, Fe^{3+}, Ni^{2+}) or mixed supply of metals (Fe^{2+} and Ni^{2+}) increased the specific hydrogenase activity by ~50%. These results suggest that *C. beijerinckii* DSM791 could be used as a potential H_2 producer. It may help to further enhance H_2 production using different industrial or agricultural wastes where glycerol and other carbon sources are present.

Introduction

Global warming, pollution caused by use of fossil fuels, along with a substantial reduction of natural gas and oil anticipates the exploration of clean and renewable energy sources. One of these sources might be dihydrogen (H_2) which has the highest energy density per mass among known fuels (142 MJ/kg) and can be produced by diverse microbes or other organisms [1,2]. H_2 is a promising alternative energy carrier as it is 'eco-friendly' and its combustion generates only water.

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Conversion of sugars, glycerol or miscellaneous organic carbon-containing industrial or agricultural wastes to H_2 either by dark- or photo-fermentation has been established, and the description and engineering of these bioprocesses have already been developed [3,4]. Co-utilization of diverse carbon sources by various bacteria has been studied extensively [5–8] but the ability of microorganisms to produce H_2 from different carbon sources and their mixtures is less clear, and therefore investigations to identify cheap and effective carbon sources that drive H_2 production are highly pertinent.

Glycerol fermentation by *Clostridium pasteurianum* was reported more than two decades ago [9], and the main fermentation end products were butanol, 1,3-propanediol (1,3-PDO), ethanol, and acetic acid. Various strains of clostridia were isolated and investigated for glycerol fermentation to 1,3-PDO as this product is widely used in the synthetic chemical industry [10,11]. With increasing industrial production of biodiesel, glycerol as the main side product (about 10% w/w) [12,13] becomes an interesting and very cheap source for production of valuable chemicals [14,15]. For this purpose, many bacterial strains have been tested that ferment glycerol and produce organic acids, ethanol, or H_2. Among them is *Escherichia coli*, which has been shown to ferment glycerol at both acidic [16–18] and alkaline [19] pHs. Crude and pure glycerol gave the same results [20,21]. Moreover, recently thermodynamic analysis of H_2 production from crude glycerol using *C. pasteurianum* was done [22]. Currently, development of H_2 production technology focuses on the use of inexpensive substrates for producing valuable fuels and chemicals from various organic acids such as formate, lactate or sugars like lactose or xylose, which can be found in miscellaneous industrial or agricultural wastes [23,24].

Most of the investigations of glycerol fermentation by clostridia have concentrated on butanol, butyrate or 1,3-PDO production pathways and further engineering of the strains for enhanced generation of these compounds [11,25,26]. Recently, due to the demand for alternative clean energy sources, several clostridial strains (*Clostridium butyricum*, *Clostridium felsineum*, *C. pasteurianum*, *Clostridium beijerinckii*) were tested for production of H_2 [27–29]. H_2 generation was examined mainly using glucose or organic acids as substrates [28,30]. Several investigations were done with newly isolated *Clostridium* strains (*C. pasteurianum* CH4) for H_2 production during glycerol fermentation, and further optimization of some external parameters like pH, temperature, and agitation rate were analyzed [28,31]. The same strain was employed also for enhanced bio-butanol production [32]. In addition, it was shown that *Clostridium* BOH_3 can be used for fermentation of agricultural residues and production of H_2 [33].

H_2 is produced by hydrogenase (Hyd) enzymes which reversibly oxidize H_2 to $2H^+$. Different strains of clostridia have [Fe-Fe] and/or [Ni-Fe] Hyd enzymes [34]. Moreover, formate dehydrogenase (FDH) is present in some *Clostridium* strains (*C. pasteurianum*, *C. beijerinckii*) but limited information is available on whether a formate hydrogenlyase (FHL) -type reaction might be involved in hydrogen production by these bacteria [34].

The main goal of the present work, therefore, was to study the ability of *C. beijerinckii* to convert glycerol to H_2. Moreover, the effects of pH and of mixtures of various carbon sources on Hyd activity and H_2 production were investigated. In addition, single or mixtures of different metals were employed to analyze whether they increase Hyd enzyme activity and thus H_2 production. This would be important for the use of crude or pure glycerol, or of glucose- and formate-containing wastes for H_2 evolution and further development of H_2 production biotechnology.

Materials and methods

Bacterial strain and growth conditions

The type strain of *C. beijerinckii* DSM791 (identical to ATCC25752) was obtained from the German culture collection of microorganisms and cell cultures (DSMZ, Germany).

The growth medium was prepared according to Diez-Gonzalez et al. [35] with modifications. It contained (per L): 1.5 g K_2HPO_4; 1.5 g KH_2PO_4; 492 mg $MgSO_4$ * 7 H_2O; 500 mg L-cysteine; 15 mg $MnSO_4$ *H_2O; 20 mg $FeSO_4$ * 7 H_2O; 1 mg resazurin; 2 mg *p*-aminobenzoic acid; 2 mg thiamine-HCl; 0.4 mg biotin; 0.5 g yeast extract. The pH was adjusted to 7.5 or 5.5 by 2 N NaOH or 2 N H_3PO_4. The medium headspace was flushed with nitrogen and the medium was autoclaved at 121 °C for 25 min. Cells were grown with different individual carbon sources or mixtures thereof: glucose – 40 mM, formate – 10 mM, glycerol – 110 mM.

Batch fermentations of bacteria were carried out in 120 ml sealed serum bottles containing 40 ml medium incubated at 37 °C during 96 h.

Preparation of cell-free extracts

Cultures were grown in 500 ml medium at 37 °C for 22–24 h and harvested in an anoxic chamber (Coy, Ann Arbor, MI, USA) by centrifuging in anoxic polypropylene centrifuge bottles at 16,270×*g* for 10 min at 4 °C using a Sorvall RC 5B centrifuge (Du Pont de Nemours, Bad Homburg, Germany). Cells were washed and re-suspended in anoxic phosphate buffer (50 mM, pH 7.5) and centrifuged as described [36]. The pellet was re-suspended in 5 ml of washing buffer, and the cells were broken by repeated passage through a cooled French-pressure cell at 137 MPa pressure under anoxic conditions.

Enzyme assays

Enzyme activities were measured by following absorption changes with a Jenway 6300 spectrophotometer (Bibby Scientific, Staffordshire, UK) connected to an analogous recorder SE 120 (Metrawatt, BBC Goerz, Vienna, Austria). Assays were performed under anoxic conditions in 1 ml rubber-stoppered cuvettes at 37 °C. One unit of specific enzyme activity was defined as 1 μmol of substrate or product per minute at 37 °C and per milligram of protein.

FDH enzyme activity was determined, as formate dependent benzyl viologen (BV) reduction [36,37]. The assay was

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