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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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A B S T R A C T

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.

Keywords:
Fermentation
Hydrogen production
Glycerol and mixed carbon substrates
pH
Hydrogenases
Clostridium beijerinckii

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

done with 10–50 μl cell-free extract; the reaction was initiated by adding 25 mM sodium formate. One unit of formate dehydrogenase enzyme activity is defined as the reduction of 1 μmol of BV per min. Hyd enzyme activity (H_2-dependent reduction of BV) was determined, as described [36,38], except that the buffer used was 50 mM phosphate buffer, pH 7.5. The reaction was started by adding 10–50 μl cell-free extract. One unit of Hyd enzyme activity corresponded to the oxidation of 1 μmol of H_2 per min.

The wavelength used was 578 nm and an E_M value of 8.600 M^{-1} cm^{-1} was assumed for reduced BV.

Analytical methods

Gas samples were taken from the headspace of sealed serum bottles and H_2 gas production was analyzed by gas chromatography (GC) with a GC 6000 (Carlo Erba, Milan, Italy) using a thermal conductivity detector, as described [36,39]. The carrier gas was nitrogen. H_2 production rate was calculated as mmol/per hour/per g cell mass (mmol/h/g cell mass). Organic compounds such as glucose, glycerol, ethanol, butanol, acetate etc. were measured by high-performance liquid chromatography (HPLC). Samples and standards were prepared by acidification with H_2SO_4 at a final concentration of 91 mM followed by centrifugation for 5 min at 16,100×*g* to remove cells. The supernatant was used for analysis and applied to the HPLC system with a 234 auto-injector (Gilson, Limburg-Offheim, Germany). Samples were separated at 60 °C with an Aminex HPX-87H ion-exchange resin (BioRad, Munich, Germany) using an isocratic mobile phase of 5 mM H_2SO_4 at a flow rate of 0.6 ml/min provided by a LC-10AT vp pump (Shimadzu, Munich, Germany). Organic compounds were detected with a refractive index detector RID-10A (Shimadzu, Munich, Germany) and the detector signal was recorded with the Shimadzu LC solution software.

The cell density was determined by measuring optical density at 600 nm wavelength (OD_{600}) using a tube spectrophotometer M107 (Camspec Analytical Instruments Ltd., Leeds, UK). Calculations of cell mass were done, as cell dry weight of OD = 1 value corresponds to 1 g of dry cells using a predetermined correlation between optical density at 600 nm wavelength and cell dry weight [40].

Protein concentrations were determined with the Bradford method [41] with bovine serum albumin as a standard.

Chemicals and data processing

All chemicals were of analytical or reagent grade quality and obtained from Carl Roth (Karlsruhe, Germany) or Sigma–Aldrich (Deisenhofen, Germany). Gases were obtained from Messer-Griesheim (Darmstadt, Germany), and Sauerstoffwerke Friedrichshafen (Friedrichshafen, Germany).

Experiments were performed at least three times and each time in triplicate. Data are presented as standard deviation of the mean and were highly reproducible with generally not more than 3% deviation. The validity of differences between experimental and control data was evaluated by Student's criteria (p) [7,18]; the difference is valid when $p < 0.01$ or less, otherwise p was represented, and $p > 0.5$ if the difference is not valid.

Results and discussion

Comparison of H_2 production by Clostridium beijerinckii *during fermentation of glycerol and carbon source mixtures*

To investigate whether *C. beijerinckii* growing on glycerol at different pHs (Fig. 1) is able to convert it to H_2, initially 110 mM glycerol was used. For comparison, glucose and various mixtures of carbon sources like glucose plus formate, glycerol plus formate and glucose plus glycerol were also analyzed to test if it is possible to enhance the H_2 production and increase Hyd enzyme activity by using different carbon source combinations. The latter were of importance as in nature usually no single carbon source is present. *C. beijerinckii* grew better on glycerol at pH 7.5 compared to pH 5.5. This might be due to optimal enzyme activity especially a high ATPase activity as well as keeping of neutral intracellular pH and maintaining a higher proton-motive force at high but not low pH; further study is required. This is likely to that the intracellular pH and transmembrane pH gradient follow medium pH, as shown for *Clostridium acetobutilicum* [42], and induction of some operons for global regulators and hydrogenases when the proton-ATPase activity is higher at high but not low pH, as shown for *E. coli* [43,44].

The H_2 production rate during fermentation of glycerol at pH 7.5 was ~3.5 mmol/h/g cell mass, which was ~1.2 fold lower

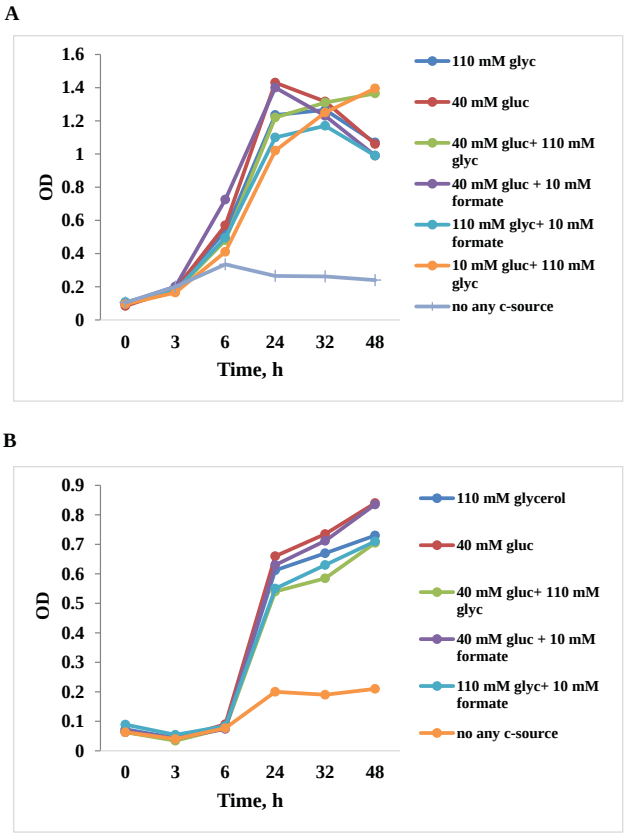

Fig. 1 – Growth of *C. beijerinckii* DSM791 at 37 °C at pH 7.5 (A) and pH 5.5 (B) during utilization of various carbon sources. For details see "Materials and methods".

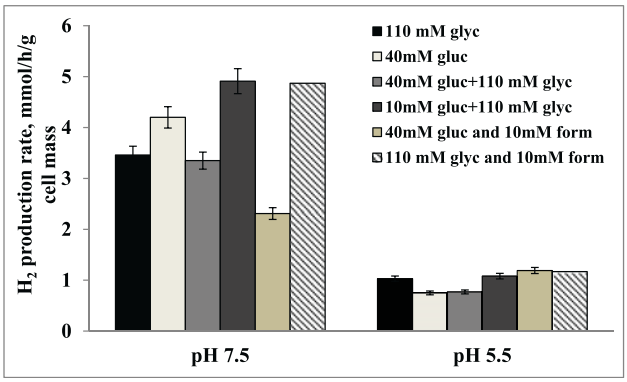

Fig. 2 – H_2 production rate by whole cells of *C. beijerinckii* DSM791 grown at pH 7.5 or pH 5.5 during utilization of single or mixtures of carbon sources. For details see "Materials and methods".

compared to growth with glucose (Fig. 2). The H_2 yield was 1.21 mol H_2/mol glycerol (Table 1) which was in the same range (1.11 mol H_2/mol glycerol) as shown for *C. pasteurianum* CH4 by Lo et al., [28]. But interestingly, a mixture of 110 mM glycerol and 10 mM formate increased the rate by ~1.4 fold compared to glycerol only. With a mixture of 40 mM glucose plus 10 mM formate, the H_2 production rate was decreased ~1.8 fold (see Fig. 2). At pH 5.5, the H_2 production rate was lower in all variations of single or mixed carbon sources. In general, during glycerol fermentation the H_2 production rate at pH 5.5 was 1 mmol/h/g cell mass and ~3.4 fold lower than at pH 7.5 (see Fig. 2). During glucose fermentation at this pH, the H_2 production rate was lower compared to glycerol (see Fig. 2). It is important to mention that many groups isolated various strains and tested for H_2 production [29,32,45–47] but they used different medium composition and various conditions (pH, temperature, agitation etc.) that is why it is difficult to compare results with other data. Especially, Lin et al. [29] showed that *C. beijerinckii* L9 can produce H_2 from glucose (2.81 mol H_2/mol-glucose), *C. beijerinckii* Fanp3 (2.52 mol H_2/mol-glucose) [45]. Masset et al. [46] showed that *C. butyricum* CWBI1009 also produced H_2 from glucose (1.69 mol H_2/mol-glucose). In our studies *C. beijericnkii* DSM 791 yielded 2.5 mol H_2/mol glucose. Recently, Morra et al. [47] reported that newly isolated *C. beijerinckii* strain has high H_2 production rate from vegetable wastes. Moreover, the conversion efficiency of H_2 produced from glycerol is 17.70% calculated as described [45]; the lower heating value of glycerol is 14,300 kJ/kg. This data are in the same range (16.7%) as shown for *C. beijerinckii* FanP3 during glucose fermentation [45]. This value means that glycerol can be competitive with glucose. Taken together it might be concluded that many strains are isolated and tested for H_2 production and stated that they are good H_2 producers. But to choose effective strains to obtain higher yields of H_2 generation and be dominant in large scale applications external conditions should be further optimized.

Fermentation products generated during growth on glycerol and various carbon source mixtures

To understand the differences in H_2 production and possible effects of fermentation end products on H_2 metabolism, soluble products were determined after growth at different pHs. During glycerol fermentation at pH 7.5, the main products were 1,3-propanediol (1,3-PDO) and butyrate (Fig. 3A). These data are in good agreement with results previously shown for *C. pasteurianum* [24,25]. Besides the main metabolites, acetate (~9 mM), formate (~8 mM) and lactate (~1 mM) were also generated. Interestingly, 110 mM glycerol was completely consumed during 48 h. These data were of interest since different data with glycerol consumption concentration and duration have been reported for different clostridia by different groups [25,48].

During glucose fermentation, the main metabolites produced were acetate and butyrate but not 1,3-PDO (Fig. 3B); among other products were formate and lactate. When cells were cultivated with a mixture of glucose (40 mM) and glycerol (110 mM) cells consumed glycerol simultaneously with glucose (Fig. 3C), and thus did not exhibit diauxic growth [45,46]. The main products were again 1,3-PDO and butyrate (see Fig. 3C), and also acetate. Only in the mixture of the above-mentioned carbon sources was ethanol detected. The fermentation end products formed are mainly in good correlation with the data from other groups [26,32,45,47]. The mixture of glucose (10 mM) and glycerol (110 mM) showed the same results as under the previous conditions with the mixture of glucose and glycerol. Also in this case, glycerol was utilized simultaneously with glucose (Fig. 3D). At pH 5.5 during fermentation of glycerol or glucose or the mixtures after 96 h, the carbon sources were not consumed completely by *C. beijerinckii*. The main product was again 1,3-PDO. Butyrate was also formed.

Hydrogenase activity of C. beijerinckii after fermentation of different carbon sources

To understand the role of Hyd enzymes in H_2 production during fermentation of different substrates and their mixtures, Hyd activity was determined. In all tested conditions

Table 1 – Comparison of H_2 yield values by different clostridia during conversion of glycerol as a carbon source.

Strain	Operation mode	Pure glycerol concentration (mM)	H_2 yield (mol H_2/mol glycerol)	References
Clostridium butyricum LMG1212t2	Batch	110	0.55	[38]
Clostridium pasteurianum CH4	Batch	110	1.11	[24]
C. pasteurianum LMG3285	Batch	110	0.6	[38]
Enterobacter aerogenes	Batch	20	1.12	[39]
Clostridium beijerinckii DSM791	Batch	110	1.21	This study

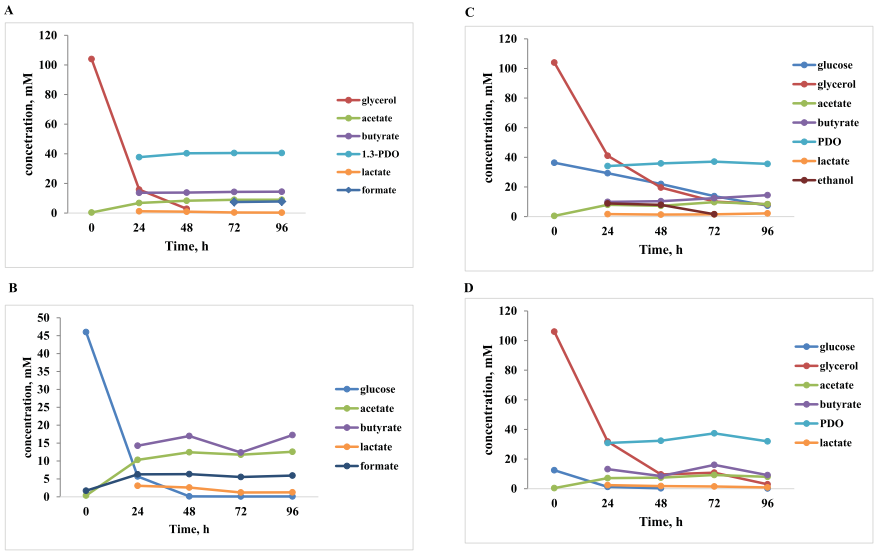

Fig. 3 – Fermentation end products of *C. beijerinckii* DSM791 grown at pH 7.5 during fermentation of glycerol (A), glucose (B), glucose (40 mM) and glycerol (110 mM) (C), glucose (10 mM) and glycerol (110 mM) (D). For details see "Materials and methods".

the Hyd activity was higher at pH 7.5 than at pH 5.5. The optimal Hyd activity at high pH can be linked to higher ATPase activity and maintaining of higher proton-motive force. This is likely to that the Hyd activity in *E. coli* is interrelated with the proton ATPase activity and maintaining of proton-motive force at high pH [49] and the proton-ATPase activity is higher at high but not low pH [43]. It is of interest that the role of hydrogenases in proton motive force generation was shown for *Clostridium acetobutylicum* [50].

When cells were grown at pH 7.5 on glucose, the Hyd activity was ~3.3 fold higher than at pH 5.5 (Fig. 4). In *C. butyricum* grown at pH 6.5 during fermentation of glucose the Hyd specific activity was 4.8 U/mg which was ~1.8 fold lower when the cells were grown on glucose and glycerol [51]. Moreover, at pH 7.5, Hyd activity of the cells grown on glycerol was ~1.4 fold higher than cells grown at the same pH on glucose. Interestingly, cells grown in the presence of glycerol plus formate at pH 7.5 showed increased Hyd activity compared to cells grown

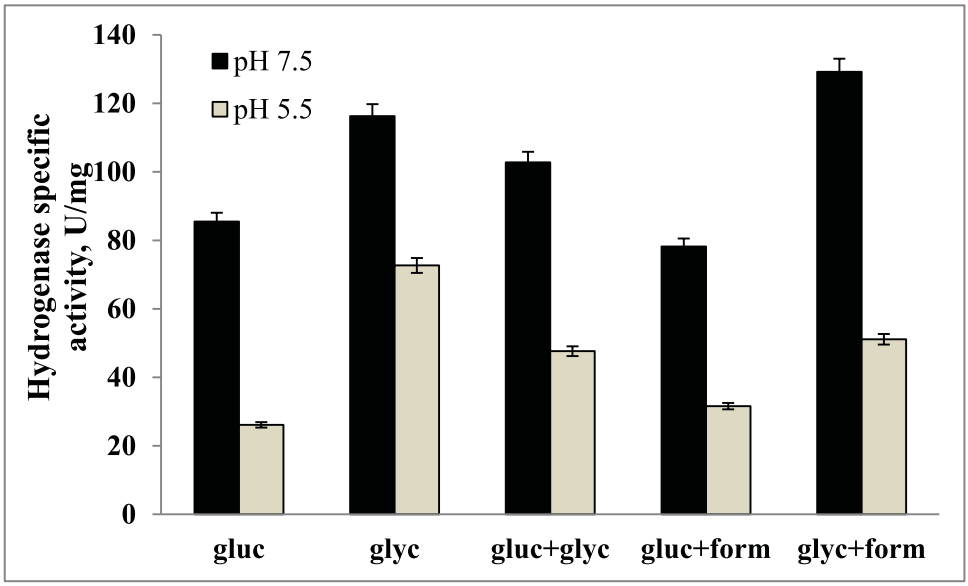

Fig. 4 – Hydrogenase activity in cell free extracts of *C. beijerinckii* DSM791 grown at pH 7.5 and pH 5.5 in the presence of various carbon sources. Cells were grown in the presence of 40 mM glucose (glu), 110 mM glycerol (glyc), 40 mM glucose plus 110 mM glycerol (gluc+glyc), 40 mM glucose plus 10 mM formate (gluc+form), 110 mM glycerol plus 10 mM formate (glyc+form). For details see "Materials and methods".

at pH 5.5. From these data it is suggested that optimal Hyd enzyme activity is determined at high pH.

Effects of various heavy metals on Hyd activity by C. beijerinckii

To reveal the role of heavy metals on Hyd activity effects of various metals (Fe^{2+}, Fe^{3+}, Ni^{2+}) were studied. These metals might affect activity of [Ni-Fe] or [Fe-Fe] Hyd enzymes [52]. Indeed, in cells grown on glucose at pH 7.5, Hyd enzyme activity was increased ~1.3 fold if 0.1 mM Fe^{3+} was added in the assays (Fig. 5A). No effect was observed for Fe^{2+} at any concentration.

When the cells were grown at pH 7.5 on glycerol plus formate the addition of Fe^{2+} (0.01 mM) stimulated Hyd activity by ~1.3 fold. The same effect was obtained when Fe^{3+} (0.5 mM) or Ni^{2+} (1 μM) was added in the assays (see Fig. 5A and B). As the results above showed that single Fe^{2+} (0.01 mM) and Ni^{2+} (1 μM) increased Hyd activity the mixture of these metals in the same concentrations were studied, and Hyd activity was increased by ~50% compared to the cell-free extracts only or by ~25% compared to the assays with single metals (Fig. 6).

The same assays were performed at pH 5.5 in the presence of different metals when cells were grown during fermentation of single or mixed carbon sources. In general, no stimulatory effects were observed with any of the metals in various concentrations. These results suggest that [Ni-Fe] and [Fe-Fe] hydrogenases are active in *C. beijerinckii* DSM791 at pH 7.5. Probably, they have a role in maintaining of proton motive force at this pH. The effects of various metals on H_2 production would be determined after optimization of the other conditions (pH, concentration of carbon sources and their mixtures) in a separate study.

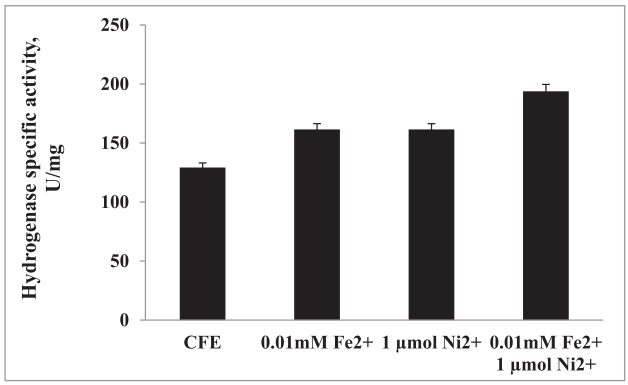

Fig. 6 – Hydrogenase activity in cell free extracts of *C. beijerinckii* DSM791 grown at pH 7.5 in the presence of glycerol and formate. In the assays single or mixtures of different metals (Fe^{2+} and Ni^{2+}) were added. For others see legends to Fig. 4.

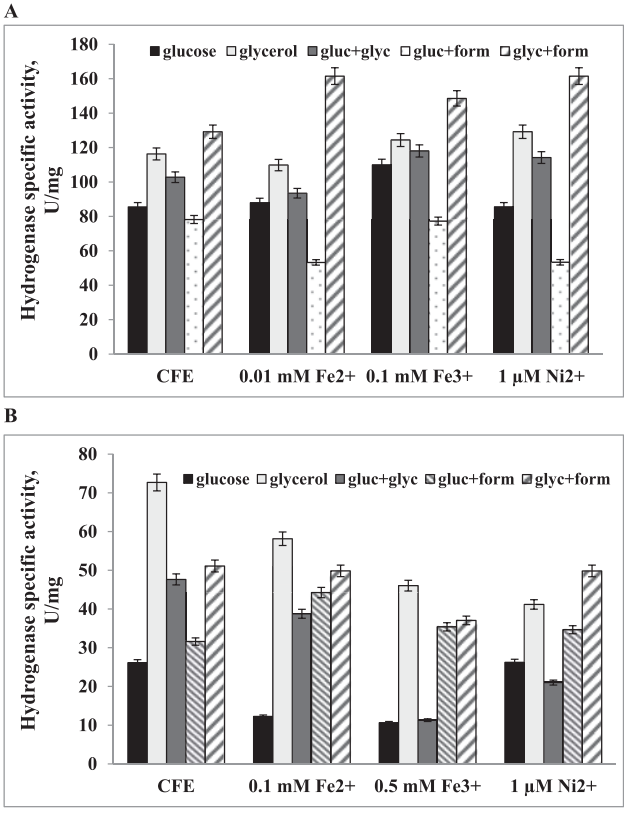

Fig. 5 – Hydrogenase activity of *C. beijerinckii* DSM791 grown at pH 7.5 (A) and pH 5.5 (B) in the presence of various carbon sources. In the assays different metals were added. For others see legends to Fig. 4.

Formate dehydrogenase activity during fermentation of various mixtures of carbon sources

H_2 can be produced from formate disproportionation by FHL; the latter consists of FDH and Hyd [53]. As Hyd activity was determined, it would be of interest to detect if FDH is active in the cells grown during fermentation of various single carbon sources or their mixtures. Indeed, in cells of *C. beijerinckii* grown on glycerol, glycerol plus formate, and glucose plus formate at pH 7.5, FDH activity was higher than that at pH 5.5 (Fig. 7). Moreover, FDH activity was increased by ~50% when the cells were grown on glycerol plus formate, compared to cells grown on glycerol only at pH 7.5 (see Fig. 7). The same

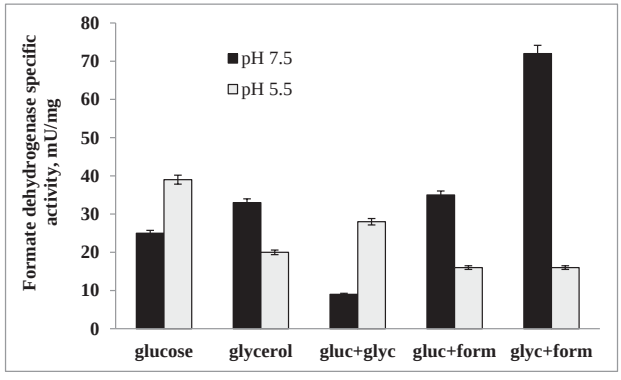

Fig. 7 – Formate dehydrogenase activity in cell free extracts of *C. beijerinckii* DSM791 grown at pH 7.5 and pH 5.5 in the presence of various carbon sources. For others see legends to Fig. 4.

stimulatory effect of formate on FDH activity was observed when the cells were grown on glucose plus formate. But surprisingly, stimulation by formate was not observed at pH 5.5. These results confirm the data obtained for Hyd activity that the cells showed FDH higher activity at pH 7.5 than at pH 5.5. This might explain less H_2 production and lower consumption of glycerol or other carbon sources by *C. beijerinckii*. Thus, FDH activity has been determined to depend on pH and carbon source, especially formate. As a substrate, formate might directly affect FDH and increase its activity. Besides, it could be suggested that FDH interacts with Hyd enzymes forming FHL complex at pH 7.5 which requires special study. This interaction might also affect FDH activity if any. The situation of FDH activity with *C. beijerinckii* seems to be likely to that for *E. coli* when different FDH and its complexes with Hyd have been proposed [54].

Conclusions and significance

The results obtained point out that *C. beijerinckii* DSM791 is an effective strain for the conversion of glycerol to H_2. Moreover, in the presence of sugar (glucose) this strain simultaneously utilized glycerol. At pH 7.5 glycerol utilization was higher compared to pH 5.5, which had impact on Hyd enzyme activity. Adding a mixture of 0.1 mM Fe^{2+} and 1 μM Ni^{2+} increased the Hyd enzyme activity by ~50% at pH 7.5 when cells were grown in the presence of glycerol plus formate. The other important finding was that growth of *C. beijerinckii* in a mixture of carbon sources used did not impair H_2 production. This would be important when considering using the organic wastes resulting from the industrial scale production of biofuels.

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