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Hydrogen Production from Paper Sludge Hydrolysate

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Abstract

The main objective of this study was to develop a system for the production of "renewable" hydrogen. Paper sludge is a solid industrial waste yielding mainly cellulose, which can be used, after hydrolysis, as a feedstock in anaerobic fermentation by (hyper)thermophilic organisms, such as *Thermotoga elfii* and *Caldicellulosiruptor saccharolyticus*. Tests on different medium compositions showed that both bacteria were able to produce hydrogen from paper sludge hydrolysate, but the amount of produced hydrogen and the requirement for other components differed. Hydrogen production by *T. elfii* strongly depended on the presence of yeast extract and salts. By contrast, *C. saccharolyticus* was less dependent on medium components but seemed to be inhibited by a component present in the sludge hydrolysate. Utilization of xylose was preferred over glucose by *C. saccharolyticus*.

Index Entries: Hydrogen production; paper sludge; *Thermotoga elfii*; *Caldicellulosiruptor saccharolyticus*.

Introduction

The Earth's climate has changed because of human activities. Since the beginning of the industrial revolution, atmospheric concentrations of the greenhouse gases—carbon dioxide, methane, and nitrous oxide—have increased continuously. Transportation specifically contributes to global warming through the burning of gasoline and diesel produced from fossil feedstock. The use of noncarbonaceous fuels, produced from

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renewable feedstock, reduces greenhouse gas emission, during fuel production and fuel combustion.

The idea of using hydrogen as a fuel is not a novel one, and interest in hydrogen production has grown in recent years. The major advantage of energy from hydrogen is the lack of polluting emissions since the utilization of hydrogen for energy production, either via combustion or via fuel cells, results in pure water. Renewable hydrogen can be produced from biomass by either gasification or fermentation *(1)*.

At present, one of the greatest drawbacks to production of renewable hydrogen is cost. The use of cheap, renewable raw materials, such as agricultural or industrial wastes or byproducts, could reduce the cost.

In Hungary, 50,000 t of paper sludge is produced annually. It is a solid industrial waste, arising from the pulping and paper-making industries, yielding mainly cellulose. Landfilling is the most preferred handling option for paper sludge, but costs have risen dramatically over the past decade and space is also limited *(2)*. However, companies from various industrial sectors have special responsibilities and tasks, according to their fields, in protecting and developing the environment. This forces the companies to find new solutions. Technologies for the production of valuable products would be attractive. Paper sludge, after being hydrolyzed, could be used as a renewable feedstock for hydrogen production during anaerobic fermentation by thermophilic microorganisms.

Several (hyper)thermophilic microorganisms that convert sugars to hydrogen, carbon dioxide, and organic acids at the theoretical efficiency of 4 mol of hydrogen/mol of glucose consumed have been described *(1)*. Members of the order *Thermotogales* were first found in natural ecosystems associated with active volcanism *(3)*. *Thermotoga elfii*, which was isolated from an African oil field, is a thermophilic, glucose-fermenting, strictly anaerobic bacterium, and it is able to grow on different carbohydrates *(4)*. *Caldicellulosiruptor saccharolyticus*, originating from New Zealand thermal springs, is an obligate anaerobic, extremely thermophilic, cellulolytic bacterium *(5)*. The present study addresses the requirements of *T. elfii* and *C. saccharolyticus* for growth and hydrogen production from paper sludge hydrolysate.

Materials and Methods

Microorganisms and Culture Media

Thermotoga elfii DSM 9442 and *Caldicellulosiruptor saccharolyticus DSM 8903* were obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen.

The medium for growth of *T. elfii* contained per liter of demineralized water: 1.0 g of NH_4Cl, 0.3 g of K_2HPO_4, 0.3 g of KH_2PO_4, 0.2 g of $MgCl_2 \cdot 6H_2O$, 0.1 g of $CaCl_2 \cdot 2H_2O$, 0.1 g of KCl, 10 g of NaCl, 0.5 g of Na-acetate, 0.5 g of cysteine-HCl, 4 g of yeast extract, 0.5 mg of resazurin, 20 mL of 10% Na_2CO_3, and 10 mL of trace element solution.

The trace element solution contained per liter: 1.5 g of nitrilotriacetic acid, 3 g of $MgSO_4 \cdot 7H_2O$, 0.5 g of $MnSO_4 \cdot 2H_2O$, 1 g of NaCl, 0.1 g of $FeSO_4 \cdot 7H_2O$, 0.18 g of $CoSO_4 \cdot 7H_2O$, 0.1 g of $CaCl_2 \cdot 2H_2O$, 0.18 g of $ZnSO_4 \cdot 7H_2O$, 10 mg of $CuSO_4 \cdot 5H_2O$, 20 mg of $KAl(SO_4)_2 \cdot 12H_2O$, 10 mg of H_3BO_3, 10 mg of $Na_2MoO_4 \cdot 2H_2O$, 25 mg of $NiCl_2 \cdot 6H_2O$, and 0.3 mg of $Na_2SeO_3 \cdot 5H_2O$.

The medium used for cultivation of *C. saccharolyticus* contained per liter of demineralized water: 0.9 g of NH_4Cl, 1.5 g of K_2HPO_4, 0.75 g of KH_2PO_4, 0.4 g of $MgCl_2 \cdot 6H_2O$, 0.9 g of NaCl, 2.5 mg of $FeCl_3 \cdot 6H_2O$, 1g of yeast extract, 0.75 g of cysteine-HCl, 0.5 mg of resazurin, and 1 mL of trace elements solution (SL-10).

The trace element solution, SL-10, contained per liter: 1.5 g of $FeCl_2 \cdot 4H_2O$, 70 mg of $ZnCl_2$, 100 mg of $MnCl_2 \cdot 4H_2O$, 6 mg of H_3BO_3, 190 mg of $CoCl_2 \cdot 6H_2O$, 2 mg of $CuCl_2 \cdot 2H_2O$, 24 mg of $NiCl_2 \cdot 6H_2O$, 36 mg of $Na_2MoO_4 \cdot 2H_2O$, 15 mg of Na_2WO_4, 15 mg of $Na_2SeO_3 \cdot 5H_2O$, and 10 mL of 25% HCl.

The medium was flushed with N_2 for 15 min and sterilized by autoclaving at 120°C for 20 min at 1.2 bar. Sterile solutions of salts, phosphate, yeast extract and trace elements were added separately after sterilization. Prior to inoculation, the pH was adjusted to 7.2 with 1 *M* KOH or 1 *M* HCl.

Preparation of Hydrolysate

As carbon and energy source, glucose or paper sludge hydrolysate was used. The paper sludge (generally 45% carbohydrate content based on dry matter; dry matter content is 60%) originated from Dunapack Pulp and Paper Mill, Dunapack Paper and Packagings, Hungary. Large-scale hydrolysis was performed at a substrate concentration of 4% (w/v) in a pH- and temperature-controlled 31-L Braun fermentor. The paper sludge was suspended in water and the pH was set to 4.8 by the addition of concentrated H_2SO_4. The slurry was sterilized at 121°C for 20 min. After cooling the mixture to 50°C, 15 filter paper units (FPU)/g of dry matter (DM) of Celluclast 1.5L and 15 IU/g of DM of Novozym 188 were added to hydrolyze the cellulose. The final paper sludge hydrolysate contained 12.8 g/L of glucose and 2.4 g/L of xylose after 48 h of hydrolysis. The hydrolysate was pretreated by mixing with 3-morpholinopropane sulfonic acid (MOPS) (10 m*M*) or phosphate buffer (the concentrations of phosphates were the same as used in the culture media) to a final pH of 7.2, and cleared by centrifugation (10 min, 13,200*g*) prior to the addition of other components.

Fermentation Assays

Cultures were grown in duplicate in anaerobic 100-mL serum bottles with 30-mL volumes. Active cultures for inoculation were prepared by growing *T. elfii* and *C. saccharolyticus* for 72 h at 65ºC and 24 h at 70ºC, respectively.

Paper sludge hydrolysate was diluted to initial glucose and xylose concentrations of 9.2 and 1.9 g/L, respectively. The flasks were incubated under different temperatures: 65°C for *T. elfii* and 70°C for *C. saccharolyticus*.

Flasks were periodically sampled during fermentation for determination of hydrogen production, soluble sugar and organic acid contents, optical density, and pH.

Analytical Procedures

The optical densities of the cultures were determined at 580 nm on an Ultrospec 2000 Spectrophotometer.

Glucose, xylose, and organic acids were analyzed by high-performance liquid chromatography with differential refractometry detection and a Shodex ionpack KC811 column at 80°C. H_2SO_4 (3 m*M*) was used as eluent at a flow rate of 1 mL/min. The samples were 1:1 diluted in 1 *M* H_2SO_4 with 250 m*M* propionic acid, as the internal standard, and filtered through a 0.45-μm filter prior to injection.

Hydrogen was determined by gas chromatography with thermal conductivity detection and an RVS MolSieve 5A, 60/80 mesh, 3 m × 0.125-in. column at 50°C. The temperature of the detector and the injector were 100 and 80°C, respectively. N_2 was used as carrier gas.

Statistical analyses were done using MINITAB software.

Results

Effect of Phosphate and MOPS Buffer

In the paper sludge, the main mineral components are typically kaolin and $CaCO_3$ (used for coating and/or as filler) and TiO_2 (used for whitening) *(6)*. When paper sludge hydrolysate was mixed with the culture medium used for growth of the microorganisms, probably precipitation of Ca-phosphate occurred. To avoid this precipitation, phosphate buffer was added first and the pH was adjusted to 7.2. After centrifugation, the other medium components were added to the clarified supernatant and no further precipitation occurred. In a control experiment, MOPS was used to replace the phosphate buffer.

As can be seen from Table 1, hydrogen was produced from paper sludge hydrolysate by *T. elfii* and *C. saccharolyticus*. Replacement of phosphate by MOPS buffer had no effect on glucose consumption or on hydrogen and acetate production. During fermentation, the pH decreased continuously to 5.2. Although both *T. elfii* and *C. saccharolyticus* seemed to grow well without supplementation with phosphate, in further experiments the hydrolysate was always pretreated with phosphate buffer and the precipitate was removed prior to fermentation.

Effect of Medium Composition

The medium components required for optimal hydrogen production by *T. elfii* and *C. saccharolyticus* on paper sludge hydrolysate were studied.

Table 1
Effect of Phosphate and MOPS Buffer on Glucose Consumption, Hydrogen, and Acetate Production by *T. elfii* and *C. saccharolyticus* After Growth on Paper Sludge Hydrolysate[a]

	T. elfii			*C. saccharolyticus*		
Medium	Glucose (m*M*)	H_2 (m*M*)	Acetate (m*M*)	Glucose (m*M*)	H_2 (m*M*)	Acetate (m*M*)
Phosphate	23.9	27.2	20.9	5.4	21.6	12.7
MOPS	20.8	27.1	19.8	3.1	20.5	11.5

[a]Samples were taken after 92 and 88 h, respectively. During fermentation, only glucose, hydrogen, and acetate were measured.

Table 2
Scheme for Addition of Salts, Yeast Extract, and Trace Elements to Media Used for Growth of *T. elfii* and *C. saccharolyticus*[a]

		Yeast extract	Salts[b]	Trace elements
1	glc pos cont	+	+	+
2	psh pos cont	+	+	+
3	– t.e.	+	+	–
4	– salts	+	–	+
5	– salts, t.e.	+	–	–
6	– y.e.	–	+	+
7	– y.e., t.e.	–	+	–
8	– y.e., salts	–	–	+
9	– y.e., salts, t.e.	–	–	–

[a]glc pos cont, positive control on glucose; psh pos cont, positive control on paper sludge hydrolysate; t.e., trace elements; y.e., yeast extract; 2–9, paper sludge hydrolysate as carbon and energy source; +, present; –, absent.

[b]*see* Materials and Methods (cysteine-HCl, resazurin, and Na_2CO_3 were always present).

The composition of the media is schematically presented in Table 2. To assess the effect of supplementation on fermentation by the microorganisms employed, the concentrations of carbohydrates, hydrogen, acetate, and lactate were measured. Fermentations on paper sludge hydrolysate with initial concentrations of 8.9 g/L of glucose and 1.8 g/L of xylose were compared with a fermentation on glucose medium (9.3 g/L of glucose).

Figure 1 shows the results of fermentations with *T. elfii* at 65°C. Hydrogen production by *T. elfii* on paper sludge hydrolysate supplemented with all media components was similar to hydrogen production by the positive control. No significant difference in glucose consumption and acetate production was observed. However, some lactate was produced during fermentation of paper sludge hydrolysate. Omission of trace elements from the paper sludge hydrolysate medium had no significant effect.

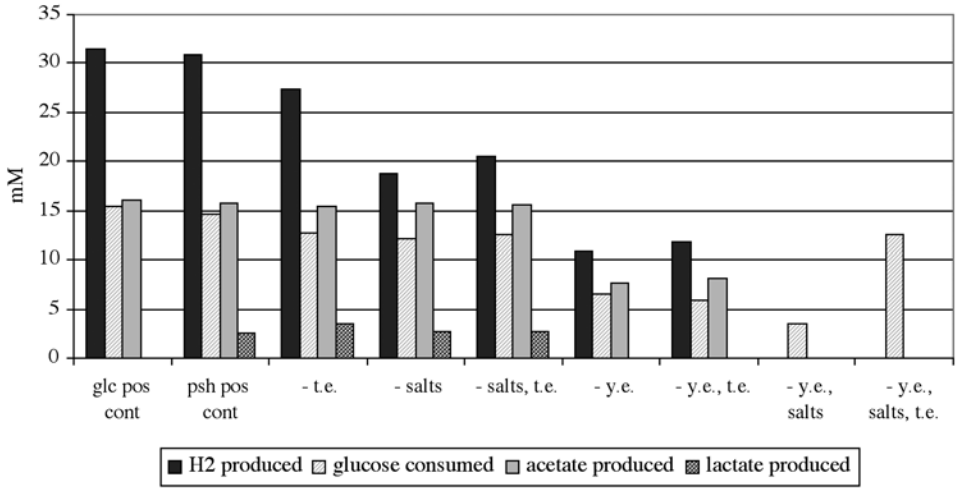

Fig. 1. Effect of different medium components on glucose consumption and hydrogen, acetate, and lactate production during fermentation of paper sludge hydrolysate by *T. elfii* after 72 h at 65°C; *see* Table 2 for abbreviations.

In the absence of all salts (except cysteine-HCl and resazurin) and plus or minus trace elements, hydrogen production was lower. This is not surprising since *T. elfii* is known as a halophilic bacterium. However, it is surprising that hydrogen production decreased while glucose consumption and acetate production remained the same. Other experiments showed that when either NaCl or the other salts were added to this medium, hydrogen production was partly restored but still lower compared to the complete paper sludge hydrolysate medium (results not shown). The omission of yeast extract had the greatest effect. Hydrogen production in cultures without yeast extract was much lower or absent when salts were also omitted. In the latter cultures, glucose concentration decreased even though no products were detected. At present, it is unclear whether this is owing to Maillard reactions or other nonspecific reactions. Maillard reactions have been observed before but are hardly reproducible and difficult to quantify. Lactate was only produced in paper sludge hydrolysate based medium enriched with yeast extract.

The results were supported by statistical calculations (Fig. 2). Supplementation of the medium with either yeast extract or salts had the greatest effect, and trace elements were practically without any influence. The effects of these variables on hydrogen production were independent.

A similar experiment was performed with *C. saccharolyticus*. The results are shown in Fig. 3. Hydrogen production in the control culture on glucose was comparable with hydrogen production on glucose by *T. elfii*. However, the production of hydrogen by *C. saccharolyticus* on paper sludge hydrolysate supplemented with all medium components was much lower than the positive control on glucose. In contrast to *T. elfii*, both glucose and

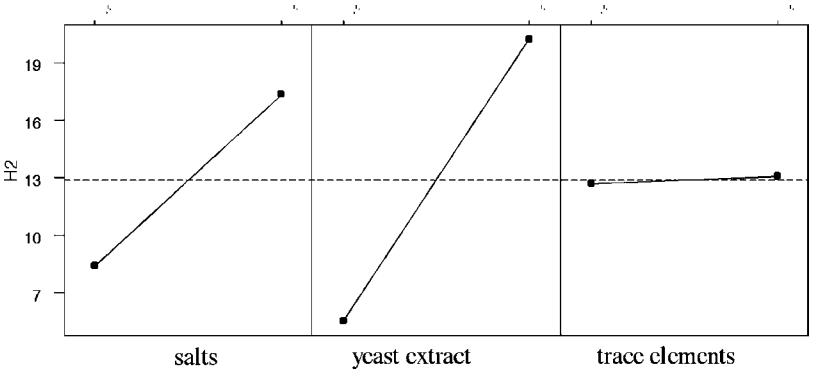

Fig. 2. Statistical analysis of effect of medium components on hydrogen production from paper sludge hydrolysate by *T. elfii*.

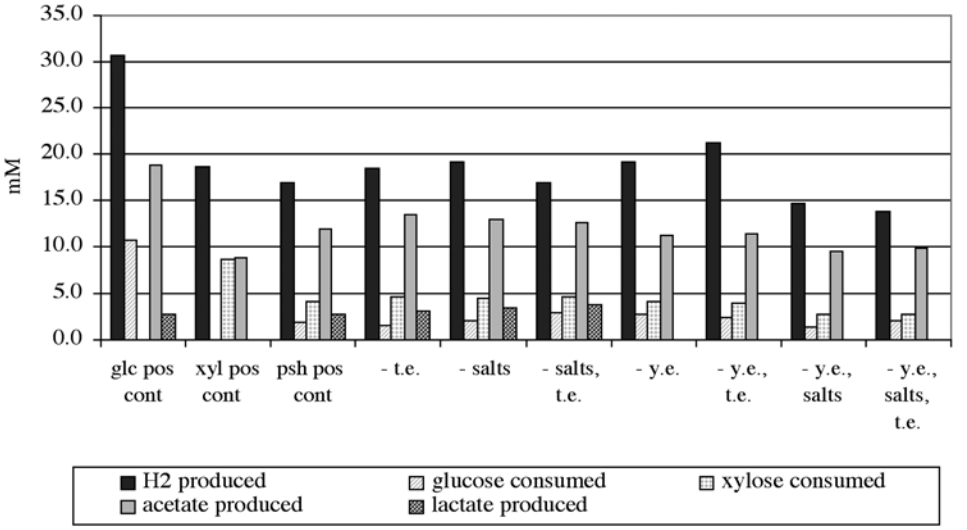

Fig. 3. Effect of different medium components on glucose and xylose consumption and hydrogen, acetate, and lactate production during fermentation of paper sludge hydrolysate by *C. saccharolyticus* after 42 h at 70°C. xyl pos cont; positive control on xylose; *see* Table 2 for abbreviations.

xylose, which are present in small amounts in paper sludge hydrolysate, were consumed by *C. saccharolyticus*. The control culture on xylose produced less hydrogen than the control culture on glucose. This could be owing to a lower consumption of xylose in this experiment and to the hydrogen yield, which is most probably lower per mol of consumed C_5 sugar (xylose) than per mol of consumed C_6 sugar (glucose). Interestingly, the omission of medium components did not further decrease hydrogen production on paper sludge hydrolysate. Thus *C. saccharolyticus*, growing on paper sludge hydrolysate, was able to produce a significant amount of hydrogen in the absence of yeast extract. During fermentation, in the presence of yeast extract, lactate production was observed (Fig. 3).

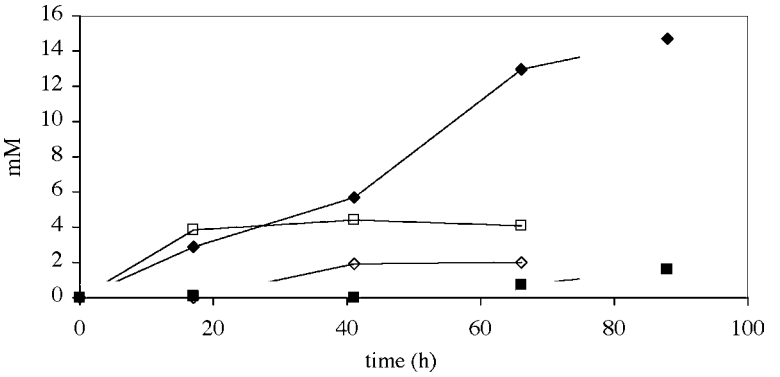

Fig. 4. Carbohydrate consumption (◇, glucose, □, xylose) during fermentation of paper sludge hydrolysate by *T. elfii* (black symbols) and *C. saccharolyticus* (open symbols).

In contrast to *T. elfii* cultures on paper sludge hydrolysate, glucose consumption by *C. saccharolyticus* was much lower (Fig. 4). Furthermore, in *C. saccharolyticus* cultures the utilization of xylose was preferred over glucose. *T. elfii* only consumed xylose at the end of the exponential phase and then only to a very small extent. However, especially for *T. elfii*, interpretation of the results was complicated because in the absence of hydrogen production, probably also in the absence of growth, xylose concentrations were increasing. It is possible that because of increased activity of xylanase or {b}-xylosidase present in the Celluclast preparation, hydrolysis of hemicellulose and derived oligomers continued, owing to the pH and high temperature during the fermentation.

Discussion

There is an abundance (50,000 t annually in Hungary) of paper sludge (industrial byproduct) with high carbohydrate content, which, after enzymatic hydrolysis, could be a substrate for hydrogen fermentation. *T. elfii* and *C. saccharolyticus* have been identified as extremely thermophilic microorganisms able to convert sugars to hydrogen, carbon dioxide, and organic acids. Therefore, the growth of these two bacteria on paper sludge hydrolysate was studied.

Both *T. elfii* and *C. saccharolyticus* could grow and produce hydrogen on paper sludge hydrolysate, but the levels of hydrogen production and the medium requirements for optimal hydrogen production differed. *T. elfii* produced a high amount of hydrogen, but needed yeast extract to do so. Moreover, because it is a halophilic bacterium, 1% NaCl was required. *C. saccharolyticus* seemed to be less dependent on additional medium components, since the hydrogen production was not stimulated by the addition of yeast extract, salts, or trace elements to paper sludge hydrolysate. In contrast to *T. elfii*, hydrogen production by *C. saccharolyticus* seemed to be

inhibited by a component present in the paper sludge hydrolysate. Both organisms produced not only acetate but also lactate, but only in a nitrogen-rich medium containing yeast extract.

The theoretical yield of hydrogen from glucose was 4 mol of hydrogen/mol of sugar. Approximately 46 and 48% of the maximum hydrogen yield was obtained with *T. elfii* growing on glucose and on paper sludge hydrolysate (based on glucose consumption), respectively. Since the theoretical hydrogen yield on xylose, a C_5 sugar, has not been determined yet, it is hard to calculate the hydrogen yields for *C. saccharolyticus*, which utilizes both glucose and xylose. The hydrogen yield found with *T. elfii* on glucose was significantly lower than previously reported *(7)*. This could be owing to the accumulated hydrogen in the closed bottles, which inhibits further production. In future studies, fermentations on a larger scale under controlled conditions and with N_2 sparging will allow more accurate determinations of hydrogen yields.

We previously observed that *T. elfii* is able to utilize amino acids for hydrogen production (results not shown). This phenomenon was also checked with *C. saccharolyticus*, but here no hydrogen production from yeast extract was observed. The utilization of yeast extract for hydrogen production also hampered the determination of an accurate mass balance. Besides this effect on obscuring the metabolic route, the dependence on yeast extract is not in favor of commercial exploitation of *T. elfii*.

According to the results we have shown, it can be concluded that both microorganisms are able to produce hydrogen from paper sludge hydrolysate. The dependence on additional nutrients was higher for *T. elfii* than for *C. saccharolyticus*. *C. saccharolyticus* seemed to be inhibited by paper sludge hydrolysate. It was clearly demonstrated that utilization of xylose by *C. saccharolyticus* is preferred over glucose. *T. elfii* only consumed xylose at the end of the exponential phase and to a very small extent. Optimization of hydrogen production by extreme thermophiles from paper sludge will be further investigated in future studies.

Acknowledgments

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