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Yields from Glucose, Xylose, and Paper Sludge Hydrolysate During Hydrogen Production by the Extreme Thermophile *Caldicellulosiruptor saccharolyticus*

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Abstract

This study addressed the utilization of an industrial waste stream, paper sludge, as a renewable cheap feedstock for the fermentative production of hydrogen by the extreme thermophile *Caldicellulosiruptor saccharolyticus*. Hydrogen, acetate, and lactate were produced in medium in which paper sludge hydrolysate was added as the sole carbon and energy source and in control medium with the same concentration of analytical grade glucose and xylose. The hydrogen yield was dependent on lactate formation and varied between 50 and 94% of the theoretical maximum. The carbon balance in the medium with glucose and xylose was virtually 100%. The carbon balance was not complete in the paper sludge medium because the measurement of biomass was impaired owing to interfering components in the paper sludge hydrolysate. Nevertheless, >85% of the carbon could be accounted for in the products acetate and lactate. The maximal volumetric hydrogen production rate was 5 to 6 mmol/(L·h), which was lower than the production rate in media with glucose, xylose, or a combination of these sugars (9–11 mmol/[L·h]). The reduced hydrogen production rate suggests the presence of inhibiting components in paper sludge hydrolysate.

Index Entries: Hydrogen production; paper sludge hydrolysate; extreme thermophile; *Caldicellulosiruptor saccharolyticus*, glucose; xylose; carbon balances.

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Introduction

Hydrogen has been recognized as one of the potential energy carriers for a sustainable energy economy in the future providing the feedstock for fuel cells. Hydrogen made from local renewable energy resources provides a clean, CO_2-neutral energy source with the additional benefit of decreasing foreign fuel dependency. During the energy crisis of the 1970s, enormous efforts were put into work exploring possible resources for production and applications of hydrogen. Unfortunately, after the decrease in crude oil price on the world market, alternative fuels were no longer of interest *(1)*. However, new concerns about greenhouse effects owing to the utilization of fossil fuel resources have brought research and development activities targeting production of biofuels from renewable resources back to life since the early 1990s. At present more than 96% of hydrogen produced worldwide depends on fossil energy resources. Alternative routes that are cost-effective and CO_2 neutral are required. The production of hydrogen from renewable resources could be a promising alternative to the present situation *(2,3)*.

Among the sustainable alternatives—electrochemical production using renewable resources, and thermochemical or biologic conversion of biomass or photobiologic hydrogen production—the biologic production of hydrogen from biomass seems to be a very attractive technology *(1,4)*. Advantages of the anaerobic fermentative route have been pointed out since several carbohydrates containing residues and byproducts can be utilized. Various waste streams, such as sugarcane juice, corn pulp, tofu-manufacturing residues, rice straw, and bean- and curd-manufacturing waste, have been investigated for hydrogen production *(5,6)*. Woodward et al. *(7)* have estimated that in the United States alone about 7.26 million t of cellulose ends up in waste newspapers annually. Utilization of this huge amount of cellulose would yield 10.6 million m^3 of hydrogen, which could provide energy for 37 cities each with a population of 27,000 *(7)*.

Already in the late 1800s, fundamental research had established that algae and bacteria could produce hydrogen. Microbiologic hydrogen production can be carried out by various bacteria including anaerobic, facultatively anaerobic, methylotrophic, and photosynthetic species in different processes *(8)*. Anaerobic thermophilic microorganisms, such as *Thermotogales* and *Caldicellulosiruptor* species, are able to convert glucose and sucrose to hydrogen, with yields close to the theoretical stoichiometry of 4 mol of hydrogen/mol of hexose. Two moles of acetic acid and CO_2 are maximally generated as byproducts *(9,10)*. Van Ooteghem et al. *(11)* have reported that most members of *Thermotogales* tolerate moderate amounts of oxygen with no apparent decrease in hydrogen production.

Thermophilic microorganisms are able to utilize a wide range of organic wastes, such as residues from the food-processing industry *(12)*. Paper sludge is another industrial waste with relatively high cellulose content, which could provide for an interesting feedstock after, e.g., enzymatic

hydrolysis. As raw material, paper sludge is very attractive in terms of economy, because presently it is deposited in areas such as landfills. For this reason the contribution of raw material cost to the overall hydrogen production cost will become very small. However, to judge whether the process economy using paper sludge is favorable, detailed economic analysis is required especially in view of the cost deposition of inorganic residues present in the paper sludge. In addition, utilization of the fermentation byproduct acetic acid is another issue that needs to be taken into account. This could be done by integration of a photofermentation involving photosynthetic bacteria capable of producing hydrogen from organic acids *(13)*. This way an economically advantageous process can be established in which material losses are minimized *(14)*. In a previously published work, nutritional requirements of two hydrogen-producing bacteria, *Thermotoga elfii* and *Caldicellulosiruptor saccharolyticus* were investigated in small-scale experiments using paper sludge hydrolysate. It was shown that the halophilic *T. elfii* needed sodium chloride in a concentration as high as 1% in order to produce hydrogen. Furthermore, a considerable amount of yeast extract was necessary to achieve good hydrogen yields. On the other hand, *C. saccharolyticus* was much less affected by nutrient supplementation, and hydrogen production was not greatly influenced by the addition of yeast extract, salts, or trace elements *(15)*.

In this article, we present our findings on hydrogen production by the extreme thermophilic *C. saccharolyticus* using paper sludge hydrolysate in a controlled laboratory fermentor, which allowed accurate determinations of hydrogen yields and production rates.

Materials and Methods

Preparation of Inoculum

A culture of *C. saccharolyticus* was grown in 100-mL sealed anaerobe serum flasks containing 30 mL of culture medium consisting of 0.9 g/L of NH_4Cl, 1.5 g/L of K_2HPO_4, 0.75 g/L of KH_2PO_4, 0.4 g/L of $MgCl_2{\cdot}6H_2O$, 0.9 g/L of NaCl, 2.5 mg of $FeCl_3{\cdot}6H_2O$, 1 g/L of yeast extract, 0.75 g/L of cysteine-HCl, and 0.5 mg of resazurin. Prior to sterilization at 120°C for 20 min, the medium was flushed with nitrogen gas for 15 min. After sterilization, 0.4 mL of sterile 300 g/L glucose or xylose—when the fermentation was on xylose—solution was aseptically added. The medium was also supplemented with 1 mL of sterile trace element solution containing 1.5 g/L of $FeCl_2{\cdot}4H_2O$, 70 mg/L of $ZnCl_2$, 100 mg/L of $MnCl_2{\cdot}4H_2O$, 6 mg/L of H_3BO_3, 190 mg/L of $CoCl_2{\cdot}6H_2O$, 2 mg/L of $CuCl_2{\cdot}2H_2O$, 24 mg/L of $NiCl_2{\cdot}6H_2O$, 36 mg/L of $Na_2MoO_4{\cdot}2H_2O$, 15 mg/L of Na_2WO, 15 mg/L of $Na_2SeO_3{\cdot}5H_2O$, and 10 mL/L of 25% HCl. The pH of the medium was adjusted to 7.2 by adding a sterile solution of either 1 *M* KOH or 1 *M* HCl. After 1 d of incubation at 70°C, the culture of *C. saccharolyticus* was used for fermentations.

Table 1
Cultivation Conditions for Hydrogen Production by *C. saccharolyticus* Using Various Carbon Sources[a]

Condition	Description
A	10 g/L glucose as carbon source, 10% inoculum
B	10 g/L xylose as carbon source, 10% inoculum
C	6.6 g/L glucose and 1.8 g/L xylose as carbon source, 10% inoculum
D1	PS hydrolysate as carbon source, 20% inoculum
D2	PS hydrolysate as carbon source, 20% inoculum
E	PS hydrolysate as carbon source, 20% inoculum, only yeast extract added

[a] PS, paper sludge.

Preparation of Paper Sludge Hydrolysate

Paper sludge containing about 45% carbohydrates (cellulose and xylan) based on dry matter was obtained from Dunapack Paper and Packaging, Budapest, Hungary. Hydrolysis of paper sludge was performed as described previously *(15)*. The pH of the hydrolysate, containing 12.8 g/L of glucose and 2.4 g/L of xylose, was set to 7.2 by the addition of phosphate buffer containing 1.5 g/L of K_2HPO_4 and 0.75 g/L of KH_2PO_4. The neutralized hydrolysate was cleared from precipitated inorganic salts by centrifuging at 13,200*g* for 10 min. Because of the dilution resulting from the pH adjustment, inoculation, and addition of medium components, the initial concentration of glucose and xylose in the bioreactor was reduced to 6.6 and 1.8 g/L, respectively.

Hydrogen Production in a Bioreactor

Batchwise anaerobic hydrogen production by *C. saccharolyticus* was performed in a 2-L bioreactor using the medium already described. For control experiments nutrients, trace elements and carbon source (glucose, xylose, or mixture of these two) were dissolved in 1 L of deionized water (Table 1). Prior to the addition of starter culture, the production medium was heated to 70°C and sparged with nitrogen gas for 30 min in order to remove any traces of oxygen and ensure anaerobic conditions. The inoculum volume was 10% (v/v). The temperature was controlled at 70°C and the pH was maintained at neutral with 1 *M* KOH corresponding to pH 6.4 at the temperature of the fermentation. *C. saccharolyticus* was cultivated with an agitation rate of 350 rpm. Hydrogen was continuously removed by sparging with nitrogen at a flow rate of 7 L/h. Hydrogen production from the previously neutralized paper sludge hydrolysate was performed similarly to control cultivations except that nutrients and trace elements were directly added to the hydrolysate and the volume of starter culture was

Table 2
Sugar Consumption and Product Formation
for Various Cultivation Conditions Using *C. saccharolyticus*[a]

Condition	Glucose (m*M*)	Xylose (m*M*)	H_2 (m*M*)	Acetate (m*M*)	CO_2 (m*M*)	Lactate (m*M*)	Biomass (g/L)
A	51.1	—	129.5	73.8	70.7	4.0	1.7
B	—	61.7	138.1	72.7	79.3	5.6	1.6
C	34.3	14.5	113.3	63.2	64.8	15.0	1.0
D1	29.0	7.9	132.1	57.7	ND	2.7	ND
D2	37.2	6.1	84.7	49.9	ND	24.5	ND
E	37.2	6.2	87.3	41.0	ND	41.9	ND

[a]ND, not determined.

doubled (*see* Table 1). Samples were withdrawn regularly and analyzed for concentrations of biomass, glucose, xylose, and various organic acids. Calculations presented in Tables 2–4 were based on data obtained at the point at which no additional hydrogen production was achieved.

Analytical Procedures

Changes in biomass concentration throughout the fermentation process were followed by optical density (OD) measurement at 580 nm using an Ultrospec 2000 Spectrophotometer. Quantitative biomass concentration was assayed applying microbiuret cell protein determination *(16)*. Biomass concentration was expressed in grams of dry matter per liter of fermentation broth by assuming a twofold multiplication constant for microbial protein to cell mass. For carbon balance calculations, the elemental composition of *C. saccharolyticus* was assumed to be $CH_{1.8}O_{0.5}N_{0.2}$ (24.6 mg/mmol).

Prior to high performance liquid chromatography (HPLC) samples were centrifuged at 9000*g* for 5 min. Each sample was diluted 1:1 with 1 *M* sulfuric acid containing 250 m*M* propionic acid as internal standard and then passed through a 0.45-µm-pore-size filter. Glucose, xylose, lactic acid, and acetic acid were separated on an Shodex Ionpack KC811 column at 80°C using a 3 m*M* sulfuric acid mobile phase at a flow rate of 1 mL/min. For the detection of various compounds separated on the analytical column, a refractive index detector was used.

Production of hydrogen and CO_2 was monitored on-line using a CP 4900 micro Gas Chromatograph (Varian, Middelburg, The Netherlands) equipped with a thermal conductivity detector and nitrogen as the carrier gas. Hydrogen was determined on a MolSieve MSA^{HI}BF module at constant injector (60°C) and column (80°C) temperature. Analysis of CO_2 was carried out using a Pora Plot Q PPQ^{HI}BF module. The temperature of the injector and the column was kept at 80°C. For some experiments, hydro-

Table 3
Carbon Balances for Various Cultivation Conditions Using *C. saccharolyticus*

Condition	Glucose (m*M* C)	Xylose (m*M* C)	Acetate (m*M* C)	CO_2 (m*M* C)	Lactate (m*M* C)	Biomass (m*M* C)[a]	C-balance (%)[b]
A	306.6	—	147.6	70.7	12.0	68.3	97
B	—	308.5	145.4	79.3	16.8	66.7	99
C	205.8	72.5	126.4	64.8	45.0	39.0	99
D1	174.0	37.0	115.4	57.7	8.1	ND	86
D2	223.2	30.5	99.8	49.9	73.5	ND	88
E	223.2	31.0	82.0	41.0	125.7	ND	98

[a]ND, not determined.
[b]C-balance is shown as m*M* C in products as percentage of m*M* C in substrate.

gen was determined on a second gas chromatograph with an RVS MolSieve 5A (60/80 mesh, 3 m × 1/8 in.) column at 50°C. The temperature of the detector and the injector was 100 and 80°C, respectively. N_2 was used as carrier gas.

Results

Hydrogen Production on Pure Sugars

Prior to utilization of paper sludge hydrolysate for growth and hydrogen production of *C. saccharolyticus*, a set of experiments was carried out using the standard production medium described previously supplemented with analytical-grade sugars as carbon and energy source. Glucose, xylose, and a mixture of these two were applied in various concentrations as described in Table 1. The concentrations of glucose and xylose in the mixture were at the same level as found in the paper sludge hydrolysate (*see* Table 1). As shown in Fig. 1, glucose, xylose, and a mixture of these sugars supported the growth of *C. saccharolyticus*. Sugars were completely utilized by the bacterium. When xylose (condition B) was the sole carbon source present in the medium, consumption of this sugar was somewhat slower than consumption of glucose (condition A). When a mixture of sugars was used as carbon source (condition C), xylose and glucose were taken up simultaneously (Fig. 1). Utilization of sugars resulted in the formation of hydrogen, CO_2, and acetic acid, as expected (Table 2). A low amount of lactate was also produced. The molar lactate yield appeared to be higher on the sugar mixture. Biomass production was higher on single sugars compared with that on the sugar mixture. Of the available carbon source, about 22% of the consumed sugars was used for biomass production in the case of conditions A and B, while only 14% of the sugars was assimilated in cell mass for condition C (Table 3).

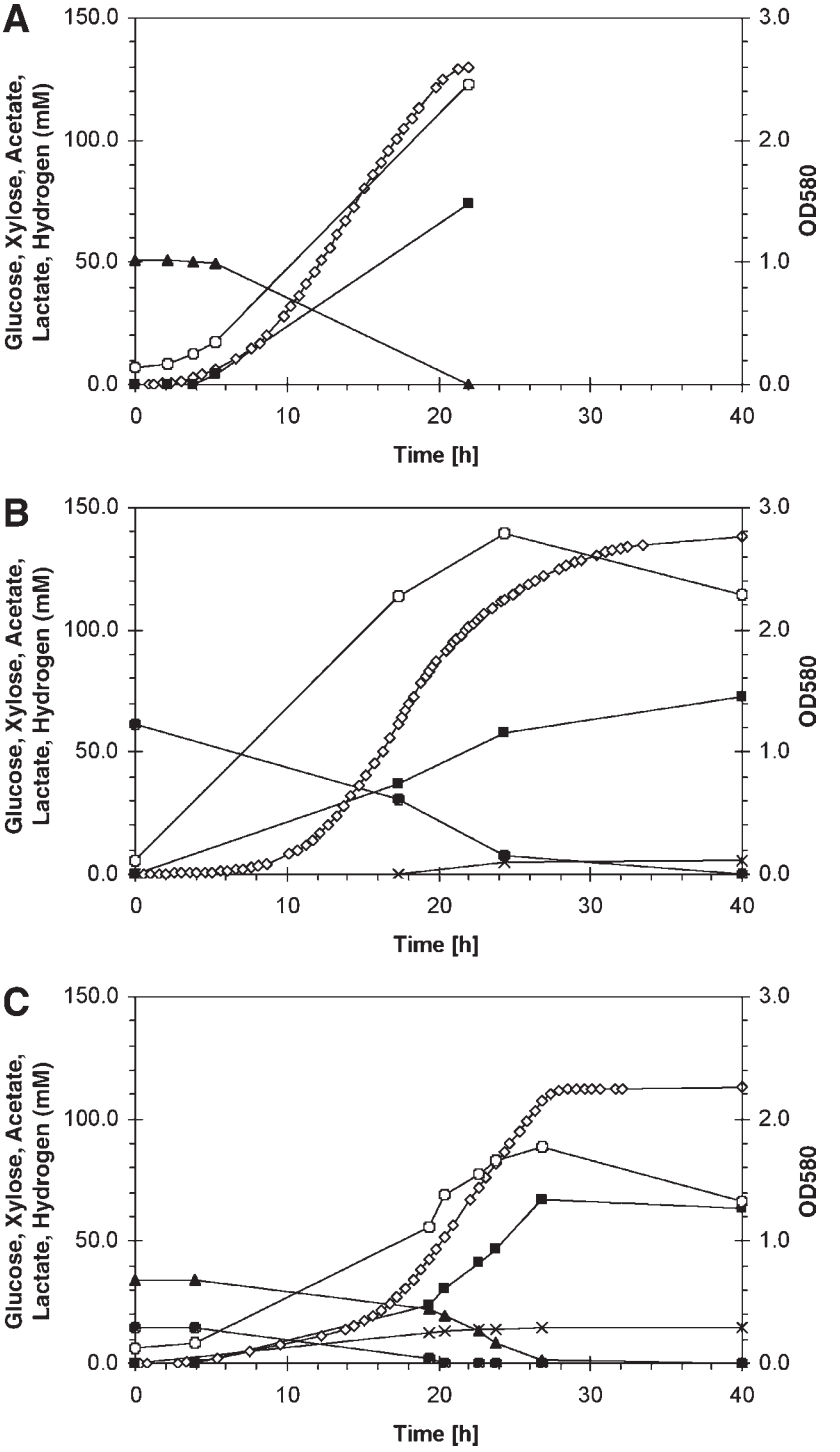

Fig. 1. Hydrogen production by *C. saccharolyticus* using: **(A)** glucose medium; **(B)** xylose medium; and **(C)** glucose and xylose medium. (—◇—), Hydrogen; (—■—), acetate; (—×—), lactate; (—▲—), glucose; (—●—), xylose; (—○—), OD580nm.

Table 4
Hydrogen and Acetate Yields as Percentage of Theoretical Maximum Yield and Maximum Hydrogen Production Rates

Condition	H_2 Yield (%)	Acetate Yield (%)	$r_{H2,max}$ (mmol/[L·h])
A	63	72	10.7
B	67	71	11.3
C	61	68	9.2
D1	94	82	5.3
D2	50	59	6.0
E	52	48	5.3

The carbon balances determined after complete consumption of the sugars are shown in Table 3. All three balances (A, B, and C) could be completed, which shows that no other products were formed from these sugars. The stoichiometry of the glucose fermentation (A) was 2.5 mol of hydrogen and 1.4 mol of acetate/mol of consumed glucose. The xylose fermentation (B) showed a stoichiometry of 2.2 mol of hydrogen and 1.2 mol of acetate/mol of xylose. Taking into account the xylose part, which was used for lactate and biomass production, since no hydrogen is produced during lactate and biomass production , 1 mol of xylose was fermented to 3.1 mol of hydrogen and 1.6 mol of acetate. These yields are close to the theoretical stoichiometry according to the following reaction:

$$3C_5H_{10}O_5 + 5H_2O \rightarrow 5CH_3COOH + 5CO_2 + 10H_2$$

In Table 4 the molar yields of hydrogen and acetic acid are presented as the percentage of the maximum theoretical yields. For conditions A and B, no major differences were observed in yields of hydrogen and acetate. The hydrogen yield was approx 65%. In the case of condition C, hydrogen and acetate yields were only slightly lower. Here, higher lactate formation was compensated by the lower biomass production. Maximal hydrogen production rates were determined for each condition. The rates were in the range of 9.2–11.3 mmol/(L·h) (Table 4).

Hydrogen Production on Paper Sludge Hydrolysate

Fermentation experiments utilizing sugars present in paper sludge hydrolysate were first carried out on complete medium as presented in Materials and Methods. Results on carbohydrate consumption and hydrogen production of two typical fermentations are shown in Table 2 (conditions D1 and D2). Acetate and lactate were the main byproducts.

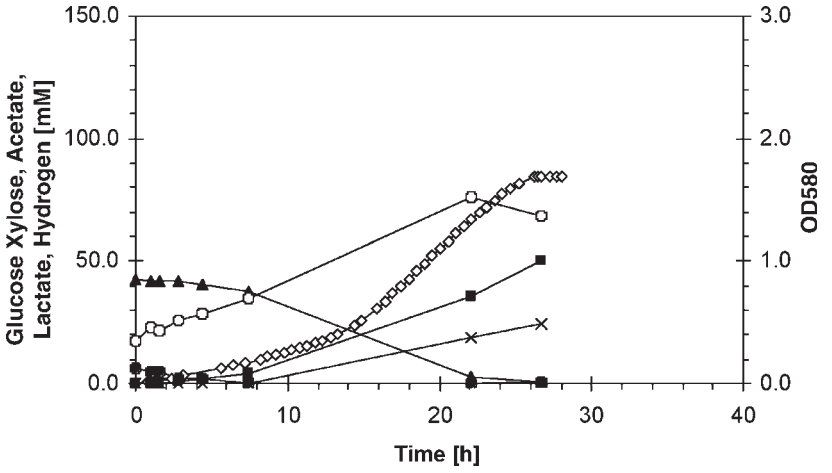

Fig. 2. Hydrogen production by *C. saccharolyticus* using paper sludge hydrolysate. (—◇—), Hydrogen; (—■—), acetate; (—×—), lactate; (—▲—), glucose; (—●—), xylose; (—○—), OD580nm.

Lactic acid formation occurred to a different extent when paper sludge hydrolysate was used. Although the same batch of hydrolysate was used for all experiments under identical conditions, lactate production was observed from the early stage (Fig. 2, condition D2) of the fermentation or lactate was only found in the fermentation broth in the very late phase of the process (condition D1).

Utilization of sugars for hydrogen production was primarily dependent on the pretreatment of the paper sludge hydrolysate. It was observed that insufficient removal of calcium ions originally present in the hydrolysate disturbed the hydrogen production of *C. saccharolyticus*. If calcium ions were not completely removed during pretreatment with phosphate buffer, precipitation of calcium salts occurred at the point of starter culture addition, thereby entirely preventing growth and hydrogen production.

Results on the carbon balances for these fermentation experiments with paper sludge hydrolysate are presented in Table 3. Interference of unidentified components in the hydrolysate with the protein content determination disallowed accurate biomass content determination, resulting in an incomplete mass balance. However, similar OD was measured as observed for the mixture of pure sugars (condition C).

Hydrogen and acetate yields on paper sludge hydrolysate were considerably higher than on pure sugars (Table 4, condition D1). When a shift in metabolism toward lactic acid production occurred (condition D2), acetate and hydrogen yields became significantly lower.

Previous experiments showed that *C. saccharolyticus* did not depend on nutrients for hydrogen production from paper sludge hydrolysate *(15)*.

Therefore, in the next experiments with paper sludge hydrolysate, all nutrients, except yeast extract and cysteine, were omitted from the production medium (condition E). Carbohydrate consumption and hydrogen and acetate production were comparable to the values found on complete medium (condition D2), where also a significant amount of the sugars was converted to lactate (Table 2). In addition, similar hydrogen and acetate yields were found and the maximum hydrogen production rate was comparable (Table 4). However, the molar carbon balance, without the contribution of the biomass, was nearly complete (Table 3), suggesting that in this case other carbon sources in the paper sludge hydrolysate were used.

Discussion

Hydrogen made from renewable energy resources provides a clean, CO_2-neutral energy source. In addition, its use would reduce fossil fuel dependency. Biologic production of hydrogen seems to be a very attractive technology for decentral hydrogen production from wet biomass *(1,4)*. Various waste streams, such as cane juice, corn pulp, tofu-manufacturing residues, rice straw, and bean- and curd-manufacturing waste, are already under investigation for hydrogen production *(5,6)*.

Small-scale fermentation experiments in sealed anaerobic serum flasks carried out with *C. saccharolyticus* have already shown that hydrogen production could be based on enzymatically hydrolyzed paper sludge *(15)*. In the present study, the potential of this hydrolysate for hydrogen production using *C. saccharolyticus* was investigated in controlled bioreactors applying nitrogen sparging to prevent product inhibition by accumulation of hydrogen in the bioreactor. Prior to using paper sludge hydrolysate as carbon source, reference fermentation experiments with pure sugars typically present in such hydrolysates were carried out for comparison (Fig. 1). This way potential inhibition by components present in organic wastes could be demonstrated *(5)*.

On glucose- and xylose-containing culture media, equal amounts of hydrogen, acetate, lactate, and biomass were produced. The yield of hydrogen based on sugars was approx 65% of the theoretical, which was found to be slightly lower than reported for sucrose (74%) *(10)*. A slightly lower yield, 61% of the theoretical was achieved, when a mixture of glucose and xylose was added to the production medium. Maximum production rates of hydrogen obtained were in the range of 9–11 mmol/(L·h) using glucose- and xylose-containing media (Fig. 1). On the other hand, a drastically reduced hydrogen production rate of approx 5.5 mmol/(L·h) was observed for fermentation on paper sludge hydrolysate, possibly owing to unidentified components in the paper sludge. For *C. saccharolyticus* cultivated on sucrose-containing medium, a maximum hydrogen production rate of 8.4 mmol/(L·h) was published in the literature *(16)*, which is very close to the rates observed in our study. *C. saccharolyticus* was able to grow directly on paper sludge hydrolysate supplemented with yeast extract and cysteine

alone. Results were comparable with those of fermentations on complete medium, showing that the requirements for other salts and trace elements is low. Analysis of the consumption of the different sugars showed that preference for xylose utilization was not as obvious as previously reported *(15)*. This may have occurred owing to the lower initial xylose concentration applied in the present study, but this theory needs further research (H. Goorissen, personal communication, 6-11-02).

On paper sludge hydrolysate the formation of lactate occurred inconsistently (Table 2). It is not clear yet what triggered the shift in the metabolism of the cells. Perego et al. *(17)* have found that at high substrate concentrations another metabolic pathway becomes active, which reduces the rates of glucose consumption and hydrogen production in *Enterobacter aerogenes*. According to van Niel et al. *(18)*, lactate and other reduced organic compounds such as ethanol or alanine were produced when reducing equivalents accumulated in the cell.

The aim of the present study was to achieve high hydrogen yields and production rates on paper sludge hydrolysate. Hydrogen production was comparable with production from pure sugars. On paper sludge hydrolysate, higher yields were achieved than on control medium. This is possibly owing to the presence of water-soluble oligosaccharides in the paper sludge hydrolysate, which were not taken into account during our calculations, but which may have been metabolized to hydrogen. The hydrogen production rate on paper sludge hydrolysate was less than observed on pure sugars. From an industrial point of view, higher volumetric production rates are advantageous. Therefore, volumetric productivity needs to be addressed in future studies. Furthermore, future research needs to address the regulation of lactate production during thermophilic hydrogen fermentation.

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