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ORIGINAL PAPER

Direct Fermentative Hydrogen Production from Cellulose and Starch with Mesophilic Bacterial Consortia

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Abstract

Hydrogen produced from lignocellulose biomass is deemed as a promising fuel of the future. However, direct cellulose utilization remains an issue due to the low hydrogen yields. In this study, the long-term effect of inoculum (anaerobic sludge) heat pretreatment on hydrogen production from untreated cellulose and starch was evaluated during repeated batch processes. The inoculum pretreatment at 90°C was not sufficient to suppress H_2 consuming bacteria, both for starch and cellulose. Although hydrogen was produced, it was rapidly utilized with simultaneous accumulation of acetic and propionic acid. The pretreatment at 100°C (20 min) resulted in the successful enrichment of hydrogen producers on starch. High production of hydrogen (1.2 l H_2/l_{medium}) and H_2 yield (1.7 mol H_2/mol_{hexose}) were maintained for 130 days, with butyric (1.5 g/l) and acetic acid (0.65 g/l) as main byproducts. On the other hand, the process with cellulose showed lower hydrogen production (0.3 l H_2/l_{medium}) with simultaneous high acetic acid (1.4 g/l) and ethanol (1.2 g/l) concentration. Elimination of sulfates from the medium led to the efficient production of hydrogen in the initial cycles – 0.97 mol H_2/mol_{hexose} (5.93 mmol $H_2/g_{cellulose}$). However, the effectiveness of pretreatment was only temporary for cellulose, because propionic acid accumulation (1.5 g/l) was observed after 25 days, which resulted in lower H_2 production. The effective production of hydrogen from cellulose was also maintained for 40 days in a repeated fed-batch process (0.63 mol H_2/mol_{hexose}).

Key words: bio-hydrogen, cellulose, enrichment, dark fermentation, repeated fed-batch

Introduction

Hydrogen is widely deemed as a promising energy carrier of the future. Currently, most of the hydrogen is delivered from the non-renewable fossil fuels – mainly through the steam reforming of methane (Sgobbi et al. 2016). In order to produce a more sustainable fuel, hydrogen production processes should avoid or minimize CO_2 emissions. Among many methods of hydrogen generation, biological hydrogen production has been of great interest in recent years (Trchounian et al. 2017).

Dark fermentation is an acidogenic decomposition of carbohydrate rich substrates. Dark fermentation has gained much interest due to its simplicity, high hydrogen production rates, and versatility of potential substrates (Łukajtis et al. 2018; Mohammed et al. 2018). Potential feedstock for hydrogen production via dark fermentation is discarded lignocellulosic biomass from agriculture, forestry and food processing. It is the most abundant raw material in nature with annual worldwide production exceeding 220 billion tons (Kumar et al. 2015). Moreover, it is renewable and has high carbohydrate content. The main component of lignocellulose is cellulose. However, lignocellulosic biomass is difficult to utilize as a feedstock for biofuel production due to its complex structure (Kumar et al. 2015; Łukajtis et al. 2018). Therefore, it may require pretreatment before biofuel production. Pretreatment of complex biomass can be done with physical, chemical, physicochemical or biological procedures (Bundhoo et al. 2015; Cai and Wang 2016). However, many pretreatment methods are not economically feasible and may produce toxic compounds such as furfural and phenolic compounds, which could inhibit hydrogen fermentation.

Complex substrates could be hydrolyzed in a non-sterile environment with mixed anaerobic consortia (like anaerobic sludge). They are the natural source of microorganisms with a wide range of hydrolytic and catabolic enzymatic activities (Gadow et al. 2012; Wang and Yin 2017). However, environmental sources for cellulolytic H_2 producing consortia often contain

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organisms that consume or prevent maximum H_2 yields, such as methanogens, homoacetogens, sulfate-reducing bacteria (SRB), propionate producers or lactic acid bacteria (Saady 2013; Bundhoo et al. 2015). Therefore, pretreatment of mixed consortia is often necessary to suppress hydrogen consuming bacteria. Its principle is that many H_2 producers like *Clostridium* and *Bacillus* can form spores, which survive the extreme pretreatment conditions. Since many H_2 consumers are non-spore formers, this process usually enriches H_2 producers and represses H_2 consumers (Saripan and Reungsang 2015). Among many pretreatment methods like aeration, heat, acid, alkaline, microwave, and UV radiation the heat treatment is the most widely used, due to its simplicity and easy control (Wang and Yin 2017). Moreover, the most efficient inoculum pretreatment method shows great variation when different feedstocks are used. In a recent study, acidic pretreatment was found the most effective during hydrogen production from xylose (Mockaitis et al. 2020). On the other hand, Yang et al. (2019) showed that the highest hydrogen yield from antibiotic fermentation residue was obtained with alkaline treatment. Therefore, it is necessary to analyze inoculum pretreatment methods for more complex substrates.

Due to the complex structure of lignocellulose biomass most reports in literature concern application of its hydrolysates for hydrogen production (Kumar et al. 2015). This approach is not economically advantageous, because an additional stage of biomass hydrolysis is required. Consolidated bioprocessing is an alternative in which cellulase production, substrate hydrolysis, and fermentation are carried out in a single step by mixed bacteria cultures that express appropriate cellulolytic enzymes (Nagarajan et al. 2019). Only a few studies were conducted with untreated cellulose (Table I). Unfortunately, yields of H_2 produced by the direct fermentation of cellulose by bacteria are still low. In a recent study, Deng et al. (2019) compared hydrogen production form raw and acid-treated grass silage. The pretreated silage led to a hydrogen yield of 68.26 ml/g_{vs}, which was 3-fold higher compared to the untreated substrate. So, the enrichment of microorganisms, which have satisfying fermentation performance, is still a daunting task. The theoretical maximum H_2 yield of dark fermentative hydrogen production is 4 mol H_2/mol_{hexose} (Dinesh et al. 2018). Recently, a high yield of 1.92 mol H_2/mol_{hexose} was obtained with co-culture of *Clostridium termitidis* and *Clostridium beijerinckii* with cellulose as a substrate (Gomez-Flores et al. 2017). The role of *C. termitidis* was hydrolysis of cellulose, while *C. beijerinckii* was responsible for the enhancement of hydrogen production. In another study, *Clostridium sartagoforme* FZ11 attained 0.53 mol H_2/mol_{hexose} from untreated microcrystalline cellulose (Zhang et al. 2015).

The dark fermentation process can be performed at thermophilic (40–90°C) or mesophilic conditions (20–40°C). Thermophilic fermentation of simple substrates is often considered better in terms of hydrogen yield (Kumar et al. 2015). However, in the case of cellulose H_2 yields are often below 1 mol H_2/mol_{hexose}. Carver et al. reported 0.35 mol H_2/mol_{hexose} during the thermophilic fermentation of microcrystalline cellulose by the mixed microbial consortium (Carver et al. 2012). On the other hand, a stable yield of 2.52 mol H_2/mol_{hexose} was maintained for 190 days of the continuous process with heat-treated anaerobic sludge and cellulose as a substrate (Jiang et al. 2015). Mesophilic processes require lower energy input since they are conducted at lower temperatures. Some batch studies achieved promising yields ranging from 1.7 to 2.09 mol H_2/mol_{hexose} (Ren et al. 2010; Bao et al. 2016). As shown in Table I, only a few studies described long term mesophilic hydrogen production from cellulose (Gadow et al. 2012).

The present work investigates the effect of inoculum pretreatment and medium composition on hydrogen production from untreated cellulose at mesophilic conditions because direct cellulose utilization represents a promising alternative to multistage processes. Two different pretreatment temperatures (90 and 100°C) were selected to prepare mixed cultures and their hydrogen fermentation performances were comprehensively assessed. The results were compared with those obtained for starch as a substrate because starch is also composed only of glucose monomers, but its hydrolysis is easier. Furthermore, the stability of enriched cultures was assessed during repeated batch processes. The effect of medium composition and pH conditions were also analyzed during long term operation. The goal of this study was to enrich the mixed bacteria culture on cellulose as the substrate and to use it in the repeated fed-batch system for mesophilic hydrogen production.

Experimental

Materials and Methods

Microorganisms and substrate. Anaerobically digested sludge originating from a municipal purification unit was a source of the microorganisms (the Central Wastewater Treatment Plant in Koziegłowy, Poznań area, Poland). The content of total solids (TS) was 43.2 g/l and volatile solids (VS) was 22.4 g/l. It was heat pretreated at 90 or 100°C for 20 minutes to suppress the activity of the hydrogen consuming bacteria and select for the spore-forming H_2 producers.

The basal fermentation medium for hydrogen production consisted of (per 1 liter): cellulose or starch 5 g, peptone 1 g, yeast extract 0.5 g, $NaHCO_3$ 2 g, KH_2PO_4

Table I
Comparison of the hydrogen production processes using untreated cellulosic material at mesophilic conditions.

Inoculum	Inoculum pretreatment	Cellulosic substrate	Conc. [g/l]	Temp. [°C]	pH initial (final)	SRE [%]	Culture conditions	Main metabolites	HPR [ml/l-h]	H_2 yield [mol H_2/mol_{hexose}]	Ref.
Sewage sludge digester	No pretreatment	Cellulose	5.0	37	5.90 (5.90)	61	Continuous	0.3	0.10	Butyrate > acetate > valerate	(Gadow et al. 2012)
Anaerobic digester sludge	70°C (30 min)	α-Cellulose	13.5[a]	37	5.5 (5.1)	NR	Batch	5	0.13	Propionate > butyrate > acetate	(Gupta et al. 2014)
Clostridium lentocellum	No pretreatment	Carboxymethyl cellulose	5	37	7.0	49	Batch	50.4	5.42[d]	Acetate > butyrate > ethanol	(Zhang et al. 2019)
Cow dung compost	No pretreatment	Microcrystalline cellulose	10	37	6.8 (4.2)	55	Batch	33	2.09	Acetate > butyrate > lactate = ethanol	(Ren et al. 2010)
Anaerobic digester sludge	No pretreatment	Carboxymethyl cellulose	4	30	7.0 (NR)	NR	Batch	NR	1.72[d]	Butyrate > acetate > ethanol > lactate	(Ho et al. 2012)
Anaerobic digester sludge	100°C (20 min)	Microcrystalline cellulose	5	32	6.0 (6.0)	60	Repeated fed-batch	2.3	0.63–1.04	Acetate > butyrate > propionate	This study
Anaerobic digester sludge	100°C (20 min)	Microcrystalline cellulose	25	32	6.5 (5.7)	82	Batch	3.94	0.97	Acetate > ethanol	This study
Clostridum sartagoforme FZ11	No pretreatment	Microcrystalline cellulose	10	35	7.0 (NR)	83.2	Batch	7.5[c]	0.53	NR	(Zhang et al. 2015)
Clostridium acetobutylicum X9 + *Ethanoigenens harbinense* B2	No pretreatment	Microcrystalline cellulose	12	37–40	6.0 (NR)	86	Batch	NR	1.7	Ethanol > acetate > butyrate	(Bao et al. 2016)
Clostridium termitidis + *Clostridium beijerinckii*	No pretreatment	Cellulose	2	37	7.2	93	Batch	2.17	1.92	Acetate > ethanol > lactate	(Gomez-Flores et al. 2017)

NR – not reported; [a] gCOD/l; [b] mmol/g_{VSS}/day; [c] ml/$g_{substrate}$/h; [d] mmol/$g_{substrate}$

3.9 g, $MgSO_4 \cdot 7H_2O$ 0.2 g, NaCl 0.1 g, $CaCl_2 \cdot 6H_2O$ 0.01 g, $FeSO_4 \cdot 7H_2O$ 0.05 g, microelements 1 ml. Microelements solution was prepared as follow (per 1 liter): $ZnCl_2$ 0.07 g, $MnCl_2 \cdot 4H_2O$ 0.1 g, H_3BO_3 0.06 g, $CoCl_2 \cdot 6H_2O$ 0.2 g, $CuCl_2 \cdot 2H_2O$ 0.02 g, $NiCl_2 \cdot 6H_2O$ 0.02 g, $NaMoO_4 \cdot 2H_2O$ 0.04 g. Microcrystalline cellulose (Aldrich – particle size 51 μm) or starch from corn (Aldrich) were used as a carbon source. The bioreactors and the medium were sterilized at 120°C for 20 min.

Procedures. The initial batch studies were carried out in triplicate in 120 ml serum bottles with a working volume of 50 ml. Bioreactors were capped with the butyl rubber stoppers and aluminum caps. After inoculation (10% v/v), headspace was flushed with argon for 15 min to create anaerobic conditions. Initial pH was set to 6.5 and then the bottles were incubated in a waterbath shaker (ELPIN 357) at 32 ± 1°C and 120 rpm. The enrichment of anaerobic sludge was performed in separate cycles. After the end of one cycle, 10% of inoculum from the bioreactor was transferred to the fresh media and the process was repeated several times.

The repeated fed-batch processes were performed in duplicates in 140 ml bioreactors (120 ml working volume). The experimental setup was described in detail in our previous publication (Zagrodnik and Łaniecki 2017). Briefly, the bioreactors were equipped with pH electrode, gaseous products outlet, inlets and outlets for replacement of the medium, and liquid samples withdrawal. Gaseous products (H_2 and CO_2) were collected in the separate graduated container. The pH inside bioreactors was maintained at 5.0 or 6.0 by automatic pH titrator, with 1 M NaOH or HCl solutions. The temperature was controlled and maintained at 32 ± 1°C with a water thermostat. The initial concentration of cellulose was 5 g/l. The basal medium with a modified concentration of cellulose (25 g/l) was used as a feed in the repeated fed-batch experiments. Initially, the bioreactors were operated in batch mode. When hydrogen production started they were switched to repeated fed-batch operation. Every 3 or 4 days 20% v/v of the medium was withdrawn from the bioreactor and the same volume was added, resulting in an average hydraulic retention time of 18.75 days and organic loading rate of 1.33 g/l/day.

Analytical methods. The samples of biogas were taken with a gas-tight syringe. Its composition was analyzed by a gas chromatograph (Varian CP-3800 with CPCarboPLOT P7 column, TCD detector). Argon was used as the carrier gas at a flow rate of 8.5 ml/min and the temperature of injector, column and detector were 115, 85 and 110°C, respectively. The content of organic compounds was determined by the HPLC technique (Dionex Ultimate 3000, ThermoScientific, SHODEX sugar column SH 1011, RI detector, 1 ml/min flow – eluent 5 mM H_2SO_4). Cellulose and starch concentration was measured according to methods described by National Renewable Energy Laboratory (NREL/TP-500-42618) (Abdullah et al. 2016).

The cumulative hydrogen gas production was determined based on the following equation:

$$V_{H,i} = V_{H,i-1} + (V_i - V_{i-1})/WV_i$$

where: $V_{H,i}$ and $V_{H,i-1}$ = cumulative hydrogen production [l/l_{medium}] at current (i) and previous (i–1) time interval; V_i and V_{i-1} = cumulative hydrogen volume [l] at current (i) and previous (i–1) time interval; WV_i = working volume of bioreactor [l] at current (i) time interval. H_2 yield was calculated as the ratio of moles of produced H_2 to moles of the substrate (expressed as moles of hexose) that have been consumed. The average hydrogen production rate was calculated by dividing the maximum volume of hydrogen produced per culture volume and the duration of the hydrogen production process.

Results and Discussion

The experiments showed that heat pretreatment of inoculum was necessary for H_2 production when starch and cellulose were used as the substrates. When pretreatment was not applied prior to inoculation, methane and CO_2 were the only gaseous products. It was carried out 90 and 100°C, because these temperatures are the most often used to enrich bacteria for hydrogen production (Wang and Yin 2017). After heat pretreatment, the produced biogas in all samples contained only hydrogen and carbon dioxide. No hydrogen was produced in reference experiments with a substrate-free medium (cellulose or starch).

Starch as a substrate with inoculum pretreated at 90°C. The initial batch tests were performed with the anaerobic sludge pretreated at 90°C. Fig. 1a presents hydrogen production during enrichment on starch. During the first cycle, only traces of hydrogen were detected. However, starch was utilized completely (Table II) with simultaneous production of acetic and butyric acid (Fig. 1a). This fact indicated microbial activity, but it was not directed towards hydrogen production. Therefore, after 12 days the bacteria in the bottle were subjected to the second treatment at 90°C and were used as an inoculum for the next cycle. This approach led to rapid hydrogen production to 0.58 l H_2/l_{medium} during the first 24 hours of the second cycle. At the same time formation of acetic and butyric acid was observed, which is characteristic for fermentative hydrogen production (Gadow et al. 2012; Łukajtis et al. 2018). Afterward hydrogen was consumed with a simultaneous increase in acetate concentration. It suggests the activity of hydrogen consuming microorganisms, such as SRB

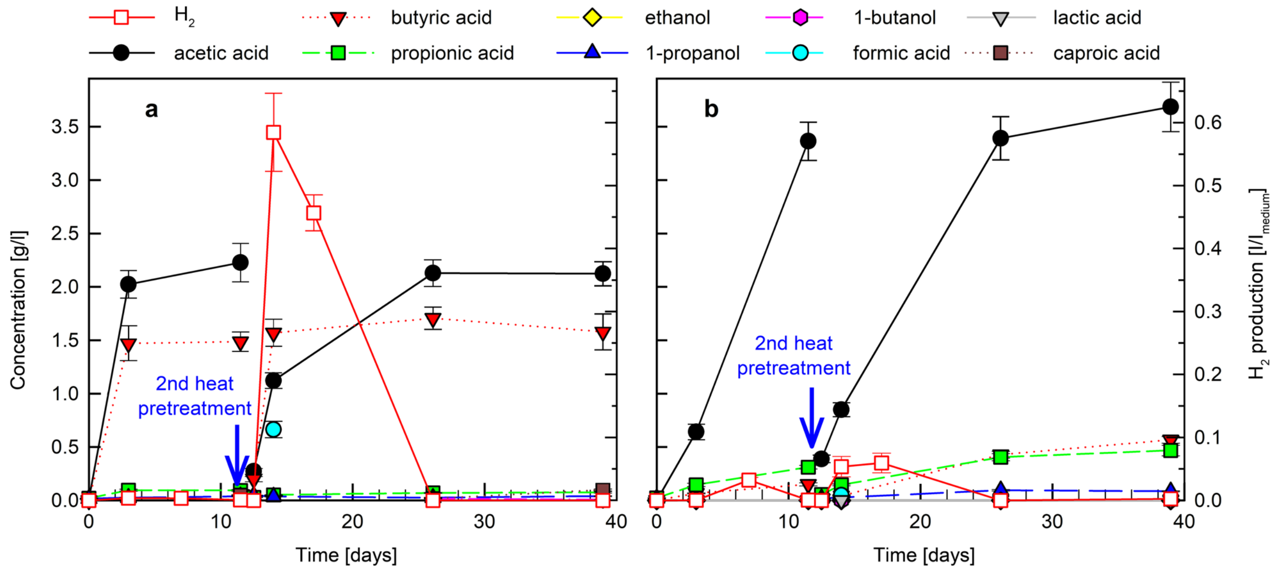

Fig. 1. Hydrogen production and the concentration of the metabolites during dark fermentation with anaerobic sludge pretreated at 90°C. Substrates: starch (a) and cellulose (b).

or homoacetogens. These two groups of bacteria are capable of hydrogen consumption and acetate production (Plugge et al. 2011; Saady 2013). Acidogenic microorganisms responsible for hydrogen production are fast growers, while hydrogen consuming bacteria are usually growing slower (Nagarajan et al. 2019). As a result, hydrogen consumption was observed after several days. The main bioreactions during hydrogen production and consumption are presented in Table III. The maximum H_2 yield (4 mol H_2/$mol_{glucose}$) is obtained when acetate is the main by-product, but this is true only when acetate is not formed in other metabolic pathways. In this

Table II

Hydrogen and CO_2 production, hydrogen yield, an average hydrogen production rate (HPR), substrate removal efficiency (SRE), and final pH in repeated batch processes from starch (S) and cellulose (C) with inoculum pretreated at 90 or 100°C.

Process	Cycle no.	Final H_2 production [l/l_{medium}]	Final CO_2 production [l/l_{medium}]	H_2 yield [mol H_2/ mol_{hexose}]	Average HPR [ml H_2/l/h]	SRE [%]	Final pH	Carbon recovered in metabolites [%]
S – 90°C	1	0	0.478 ± 0.056	0	0	100	5.70 ± 0.12	81
	2	0	0.484 ± 0.032	0	12.2 ± 1.1	100	5.16 ± 0.14	74
C – 90°C	1	0	0.42 ± 0.047	0	0.2 ± 0.1	95 ± 3	5.50 ± 0.10	75
	2	0	0.65 ± 0.038	0	0.5 ± 0.1	94 ± 3	5.71 ± 0.07	87
S – 100°C	1	0.003 ± 0.001	0.607 ± 0.040	0.01 ± 0.001	4.4 ± 0.3	100	4.60 ± 0.11	81
	2	1.175 ± 0.115	0.917 ± 0.079	1.60 ± 0.16	21.8 ± 2.0	100	5.05 ± 0.08	58
	3	1.121 ± 0.097	0.961 ± 0.050	1.53 ± 0.13	14.9 ± 1.2	100	5.90 ± 0.05	51
	4	1.167 ± 0.111	0.775 ± 0.074	1.59 ± 0.15	24.7 ± 2.2	100	5.05 ± 0.05	52
	5	1.298 ± 0.140	0.838 ± 0.086	1.77 ± 0.19	26.0 ± 1.9	100	5.05 ± 0.04	48
	6	1.200 ± 0.098	0.771 ± 0.059	1.63 ± 0.13	15.8 ± 1.2	100	5.05 ± 0.07	46
C – 100°C	1	0.093 ± 0.006	0.549 ± 0.061	0.14 ± 0.01	0.6 ± 0.1	90 ± 2	5.30 ± 0.12	75
	2	0.314 ± 0.019	0.892 ± 0.056	0.48 ± 0.03	0.5 ± 0.1	89 ± 1	5.95 ± 0.12	62
	3	0.007 ± 0.001	0.828 ± 0.097	0.01 ± 0.001	0.5 ± 0.1	95 ± 2	5.63 ± 0.11	85
	4	0.201 ± 0.023	0.456 ± 0.035	0.33 ± 0.04	0.4 ± 0.1	84 ± 3	5.40 ± 0.07	59
	5	0.253 ± 0.028	0.415 ± 0.047	0.39 ± 0.04	0.3 ± 0.1	89 ± 4	5.52 ± 0.08	50
C – 100°C (without SO_4^{2-})	1	0.585 ± 0.033	0.532 ± 0.042	0.97 ± 0.05	2.3 ± 0.2	82 ± 2	5.70 ± 0.04	56
	2	0.410 ± 0.041	0.379 ± 0.028	0.71 ± 0.07	2.6 ± 0.3	79 ± 1	4.90 ± 0.05	39
	3	0.485 ± 0.026	0.323 ± 0.022	0.93 ± 0.05	1.3 ± 0.2	71 ± 3	4.95 ± 0.09	41
	4	0.135 ± 0.014	0.337 ± 0.023	0.23 ± 0.02	1.3 ± 0.2	80 ± 2	5.02 ± 0.07	54

Table III
The main anaerobic bioreactions during hydrogen fermentation form glucose
with corresponding standard Gibbs free energies.

	$\Delta_r G°$ [kJ]	Eq. No.
Reaction		
Acetate: $C_6H_{12}O_6 + 2H_2O => 2CH_3COOH + 4H_2 + 2CO_2$	–206	1
Butyrate: $C_6H_{12}O_6 => CH_3CH_2CH_2COOH + 2H_2 + 2CO_2$	–254	2
Lactate: $C_6H_{12}O_6 => 2CH_3CHOHCOOH + H^+$	–225.4	3
Ethanol: $C_6H_{12}O_6 => 2CH_3CH_2OH + 2CO_2$	–164.8	4
Hydrogen consuming reactions		
Homoacetogenesis: $4H_2 + 2CO_2 => CH_3COOH + 2H_2O$	–104	5
Methanogenic reactions: $4H_2 + CO_2 => CH_4 + 2H_2O$	–135	6
Sulfidogenic reactions: $SO_4^{2-} + 4H_2 + H^+ => HS^- + 4H_2O$	–152.2	7
Propionate production: $C_6H_{12}O_6 + 2H_2 => 2CH_3CH_2COOH + 2H_2O$	–279.4	8

study, acetate was formed in pathways leading to the consumption of hydrogen. Consequently, no hydrogen was detected at the end of the seco nd cycle.

Starch as a substrate with inoculum pretreated at 100°C. A small volume of hydrogen (0.32 l H_2/l_{medium}) was produced during the first 24 hours in the first cycle, but then it was quickly consumed with simultaneous high production of acetate (Fig. 2). Thus, this cycle was similar to the process with bacteria pretreated at 90°C. Therefore, the bacteria culture after the first cycle was heat-treated again at 100°C for 20 minutes before the second cycle. It resulted in high hydrogen production of 1.2 l H_2/l_{medium}. Hydrogen was produced at a high rate which is characteristic of the dark fermentation process (Table II). The average HPR reached 26 ml H_2/l-h. This time hydrogen was not subsequently consumed, as it was observed when the inoculum was pretreated at 90°C, but its volume was stabilized. A similar volume of hydrogen was produced also in the next cycle. In contrast, Wang et al. (2011) observed that increasing the temperature of inoculum pretreatment from 80°C to 100°C resulted in decreased H_2 yields from glucose. In this study, the main metabolites were butyric and acetic acid (Fig. 2). Formic acid was also present at concentrations of about 0.5 g/l. These results are in line with another study, which reported butyric (2.8 g/l), acetic (0.8 g/l), and formic acid (0.2 g/l) as main byproducts of dark fermentation of glucose by heat-treated anaerobically digested sludge (Baghchehsaraee et al. 2008).

Additionally, the development of the dark color in the medium during the process suggested activity of sulfate reducers. The dark precipitate of iron sulfides is often useful to indicate the activity of SRB (Bernardez and de Andrade Lima 2015). The SRB mainly use sulfate,

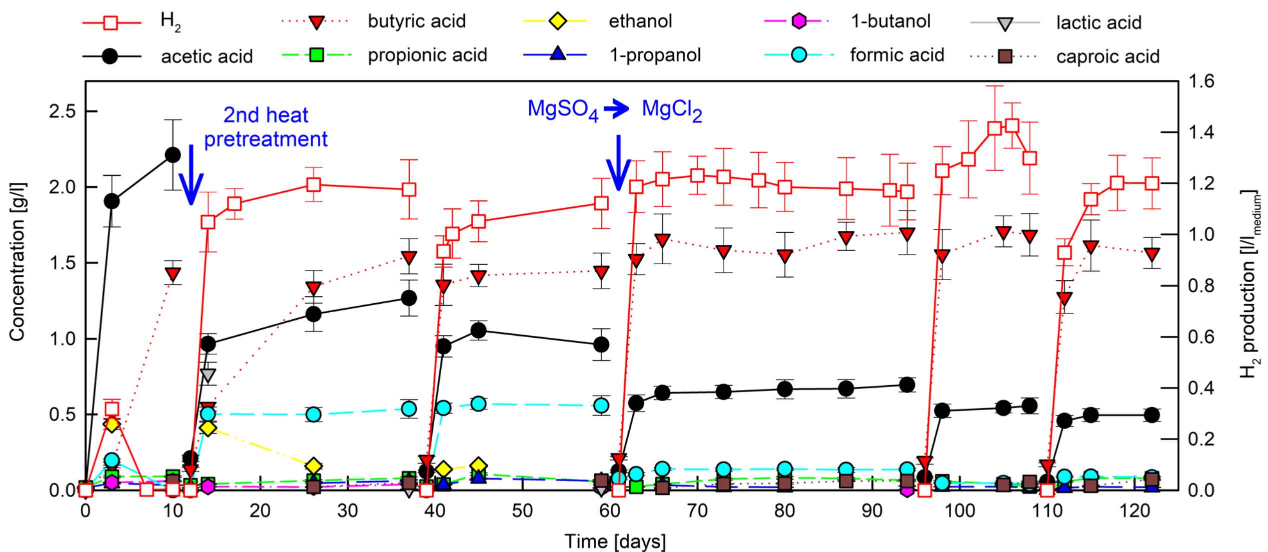

Fig. 2. Hydrogen production and the concentration of the metabolites during dark fermentation of starch with anaerobic sludge pretreated at 100°C.

the most oxidized form of sulfur, as the terminal electron acceptor. Therefore, at this point, 0.2 g/l of $MgSO_4$ in the medium was replaced with 0.165 g/l of $MgCl_2$ to reduce the concentration of this electron acceptor. This change did not have much influence on hydrogen production. However, it resulted in the change of organic acids concentration – butyric acid became dominant in the subsequent cycles with a lower concentration of acetic acid. High butyric to acetic acid ratio is characteristic of dark fermentative hydrogen production (Lin et al. 2008; Łukajtis et al. 2018). Moreover, the dark color of the bacterial culture was no longer observed. Hydrogen production remained stable in the next two cycles. Therefore, after 120 days of the process bacteria were successfully enriched for hydrogen production from starch. The complete starch consumption in each cycle was observed and H_2 yield reached 1.77 mol H_2/mol_{hexose} in the final cycles (Table II). Lin et al. (2008) obtained H_2 yield of 1.1 mol H_2/mol_{hexose} from starch with heat-treated (100°C) sludge. Similar values are often reported for mesophilic hydrogen production processes (Kumar et al. 2015; Wang and Yin 2018).

Cellulose as a substrate with inoculum pretreated at 90°C. Hydrogen production in the first cycle with cellulose was low and after 12 days H_2 was completely consumed (Fig. 1b). Moreover, high substrate utilization was observed (Table II). Acetic acid concentration reached 3.36 g/l, while lesser concentrations of propionic and butyric acid were observed (Fig. 1b). On the other hand, acetic and butyric acid concentration was similar during the process with starch (Fig. 1a). This showed that, despite the same inoculum used in both processes, in the case of cellulose utilization other microorganisms with different metabolic pathways were active. The bacteria culture after the first cycle was again heat-treated at 90°C and used as inoculum for the subsequent cycle. However, it did not result in the improvement of the volume of the produced hydrogen. The second cycle was similar to the first one, also in terms of produced metabolites and suggested the activity of hydrogen consumers. Low hydrogen production indicated that enrichment of the mixed bacteria culture in these pretreatment conditions was not possible.

Cellulose as a substrate with inoculum pretreated at 100°C. The pretreatment at 100°C led to higher hydrogen production from cellulose in the first cycle (Fig. 3a) when compared to the pretreatment at 90°C. However, H_2 yield was still low (Table II) with simultaneous acetate and propionate production (Fig. 3a). Therefore, heat treatment was repeated before the second cycle. As a result, a small volume of hydrogen (0.05 l H_2/l_{medium}) was detected at the beginning of the process, with simultaneous acetate production. H_2 production started after a lag time of 16 days and was associated with the formation of butyrate and ethanol. This indicates that the second heat treatment altered bacterial culture composition, which was correlated with a change in produced soluble metabolites. In the third cycle, only traces of H_2 were detected at the beginning, while simultaneous high acetate production indicated high activity of hydrogen consumers. The dark color of the medium in the bioreactor suggested the activity of SRB. Therefore, similarly to the process with starch, magnesium sulfate was replaced with magnesium chloride at this point. As a result in the next two cycles hydrogen production of around 0.2 l H_2/l_{medium} was observed, with concurrent lower acetic acid production. Similar results were obtained for starch, which confirms that SRB participated in the accumulation of acetate. In the presence of sulfate many fermentation products (propionate, butyrate, lactate) can be consumed by SRB with simultaneous acetate production (Plugge et al. 2011). However, when sulfate is limited, many SRB species grow fermentatively. In that case, they usually require methanogen or other hydrogen-scavengers to make this process thermodynamically favorable, because low hydrogen partial pressure is necessary for the utilization of substrates like lactate or ethanol (Baffert et al. 2019). In our experiments hydrogen concentration in medium without SO_4^{2-} was substantial. Therefore, it could be assumed that SRB activity in the sulfate-deprived medium was limited.

Cellulose consumption in each cycle was around 90% (Table II). However, generally, H_2 yields were low and did not exceed 0.5 mol H_2/mol_{hexose}, probably because other bacteria repressed hydrogen producers at this point. This experiment demonstrated that enrichment of the mixed bacteria culture is highly substrate-dependent – during enrichment on starch H_2 producing bacteria were able to outcompete the H_2 consumers.

In another experiment with cellulose as a substrate, a medium without sulfate was used from the beginning of the enrichment process. As a result, efficient production of hydrogen was observed in the initial cycles (Fig. 3b). H_2 production was six times higher than in the corresponding process with $MgSO_4$, although the utilization of cellulose was 8% lower (Table II). This indicates that minor changes in the nutrient composition can significantly alter the activity of the bacteria and the process itself. As shown in Table I, hydrogen production from cellulose was characterized by a very diversified profile of metabolites. In this study the main metabolites were acetic and propionic acid (Fig. 3b). Hydrogen production decreased in the subsequent cycles of the repeated batch process. At the same time, the concentration of propionic acid increased and became dominant in the fourth cycle. This indicates the activity of propionate producers – another group of hydrogen consuming bacteria. Thus, H_2 production by the culture enriched on cellulose was only temporary,

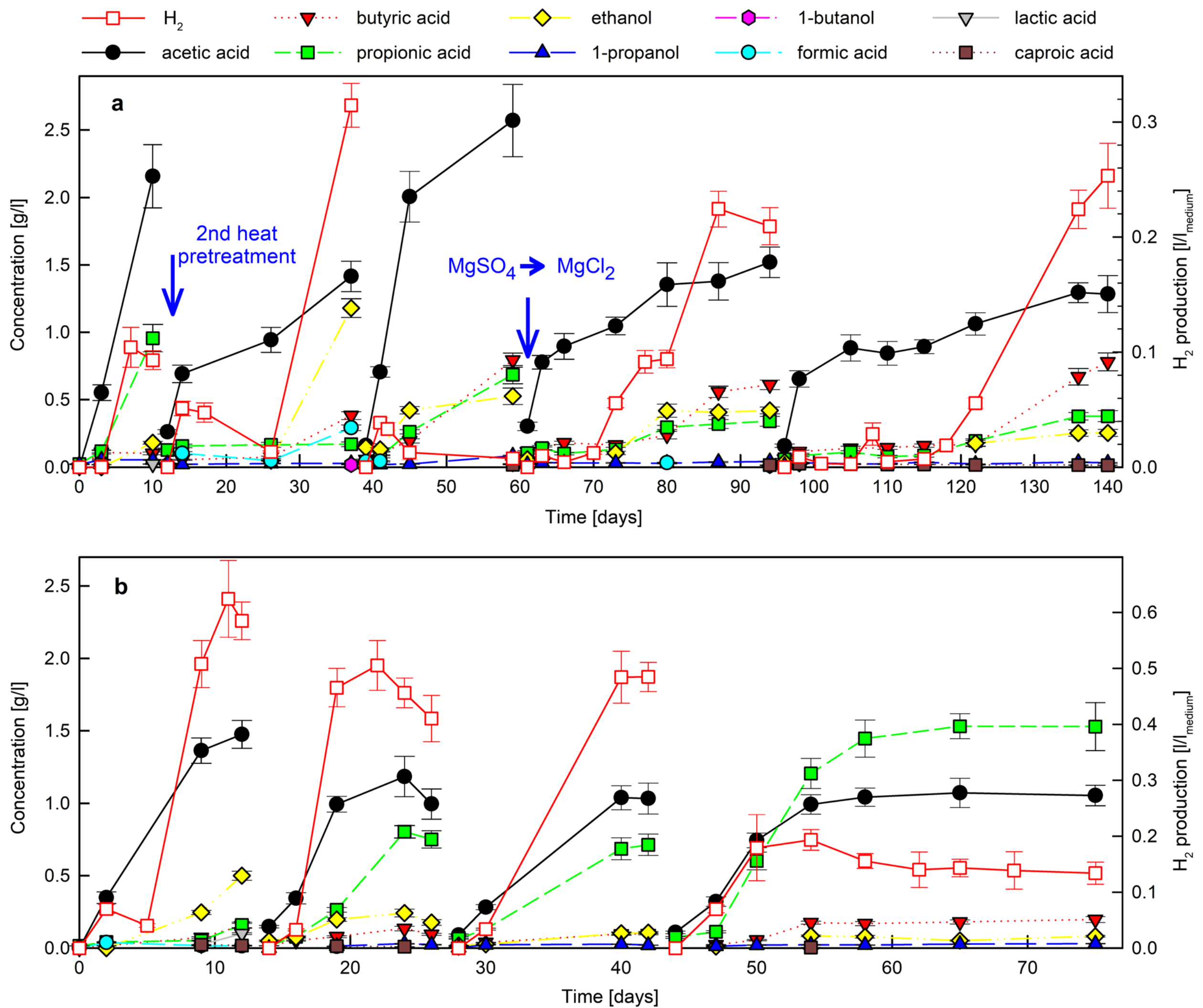

Fig. 3. Hydrogen production and the concentration of the metabolites during dark fermentation of cellulose with anaerobic sludge pretreated at 100°C (a). Medium with $MgSO_4$ replaced by $MgCl_2$ (b).

because hydrogen consuming bacteria regained their activity after several cycles.

Only a few studies analyzed the effect of heat pretreatment at different temperatures on mesophilic hydrogen production (Baghchehsaraee et al. 2008; Ravindran et al. 2010). Baghchehsaraee et al. (2008) produced hydrogen from glucose with anaerobically digested sludge pretreated at 65, 80 or 95°C. The H_2 yield for untreated sludge and sludge pretreated at 65 and 95°C were 0.43, 2.30, 1.95 mol H_2/mol$_{glucose}$, respectively. The bacterial community analysis showed that elevated temperatures reduced species diversity. On the other hand, Ravindran et al. (2010) in experiments with inoculum (forest soil) pretreated at 65, 80, 95, 105, and 120°C reported the highest H_2 production for 105°C (1.92 mol H_2/mol$_{glucose}$). When inoculum was pretreated at 105°C, the presence of additional bacterial species was detected. The authors assumed that the higher temperature was needed for efficient spore germination. These studies show that there is no agreed conclusion on the most suitable pretreatment temperature. The change of pretreatment temperature results in the different microbial communities, which might be directly responsible for the different fermentation types and hydrogen yields. This study also shows that various bacteria were involved in the H_2 production process for 90 and 100°C pretreatments, which is reflected in different metabolic profiles.

The effect of a substrate on bacteria enrichment. The results obtained showed that enrichment on starch was more effective than on cellulose. This probably resulted from the fact that starch is more susceptible to hydrolysis. Cellulose utilization for hydrogen production requires different and more complex microbial activities. Therefore, lower H_2 production from cellulose could be due to the different degradative abilities of the microbial consortium related to the different substrates. Carbon recovery in the soluble metabolites varied from 39 to 81%, depending on the process (Table II). Carbon recovery of 67% is theoretically obtained for main

fermentation reactions because four glucose carbons are recovered in acetate/butyrate, while two in the form of CO_2 (Table III – Eq. 1 and Eq. 2). Lower values indicate that more carbon was lost as CO_2 or biomass. Whereas, higher values suggest that carbon was recovered in undesirable reactions such as homoacetogenesis or propionate production. Generally, carbon recovery was higher for the processes with cellulose than with starch. Therefore, different bacteria with distinct metabolic pathways would enrich and dominate the culture on starch and cellulose. As results, different metabolites are formed. In another study with the anaerobically digested sludge pretreated at 70°C, the application of cellulose also led to the lower volume of H_2 when compared to starch (Gupta et al. 2014). It was explained by the difference in the microbial diversity of bioreactors – H_2 yields had a linear relationship with the number of observed species. Enrichment of class *Clostridia* and genera *Bacterioides* and *Parabacterioides* was found both for starch and cellulose as a substrate. On the other hand, *Lachnospiraceae* sp. was specific for cellulose cultures, while *Streptococcus* for starch cultures. Different bacteria composition could also be a reason of different H_2 and VFAs production for starch and cellulose in this study.

Other authors during studies with mixed anaerobic culture and glucose as a substrate also demonstrated that inoculum pretreatment could not permanently inhibit homoacetogenesis or methanogenesis (Shanmugam et al. 2016). In this study, no methane was detected in any experiment. The produced biogas in all of the samples contained only hydrogen and carbon dioxide. Cellulose consumption in each cycle was around 80 percent and a H_2 yield in the best cycle reached 0.97 mol H_2/mol_{hexose} (5.93 mmol $H_2/g_{cellulose}$) (Table II). Ren et al. (2010) reported the highest mesophilic batch H_2 production from microcrystalline cellulose with a yield of 2.09 mol H_2/mol_{hexose} with inoculum originating from cow dung compost. On the other hand, H_2 yield of 0.13 mol H_2/mol_{hexose} was obtained in another study with heat-shocked sewage sludge and cellulose as substrate (Gupta et al. 2014) (Table I). These discrepancies might be due to different microbial communities involved in the process. Most studies analyzed the effect of pretreatment on hydrogen production in single batch tests. Repeated batch experiments showed that it was necessary to evaluate the long-term effect of inoculum pretreatment on hydrogen production.

The repeated fed-batch process with cellulose. Finally, the repeated fed-batch experiments with cellulose as a substrate were performed. In batch experiments, bacteria culture could have experienced the nutrients deprivation periods. This could affect the bacteria enrichment process from anaerobic sludge. We assumed that more stable conditions of the repeated fed-batch process would have a positive effect on the enrichment of bacteria. For repeated fed-batch processes three types of inoculum were applied – fresh anaerobic sludge (AS1), anaerobic sludge, which was stored for 3 months at 4°C (AS2), and active bacteria culture enriched previously on starch (ES) (section 3.1). Anaerobic sludge was heat pretreated at 100°C and medium without SO_4^{2-} was applied. These processes were performed at controlled pH conditions (Fig. 4). At pH 5.0 no hydrogen production was observed for AS1 even after 30 days. Cellulose was not consumed and metabolic profile confirms the lack of microbiological activity (Fig. 5). H_2 yields with simple sugar substrates (like glucose) reported in the literature are often the highest at pH values between 5.0 and 6.0 (Dinesh et al. 2018). However, efficient cellulose hydrolysis has been reported at a pH around 7.0 (Lo et al. 2009). This suggests that pH 5.0 in the bioreactor with AS1 was too low to hydrolyze cellulose and release simple sugars for fermentation. Therefore, subsequent processes were carried out at pH 6.0.

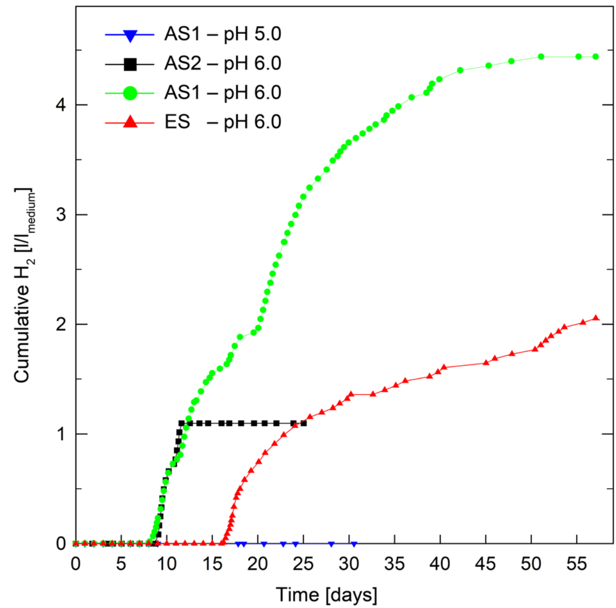

Fig. 4. Cumulative hydrogen production during repeated fed-batch dark fermentation of cellulose. AS1 – anaerobic sludge 1, AS2 – anaerobic sludge 2, ES – bacteria culture enriched on starch. Feeding days for each process: AS1 – pH 6.0 (11, 13, 16, 19, 23, 27, 31, 35, 39, 43, 47); AS2 – pH 6.0 (11, 13, 15, 18); ES – pH 6.0 (19, 23, 27, 31, 35, 39, 43, 47).

During the process with AS2 at pH 6.0 hydrogen production started after 8 days. However, hydrogen production lasted only for two feeding cycles and then no hydrogen was produced (Fig. 4). At the same time a high concentration of acetic acid was observed (Fig. 5). This indicates that hydrogen-producing bacteria were outcompeted by homoacetogens what led to the accumulation of acetic acid and inhibition of the hydrogen production. The most stable hydrogen production process was obtained when AS1 was applied at pH 6.0. Here hydrogen production lasted for about

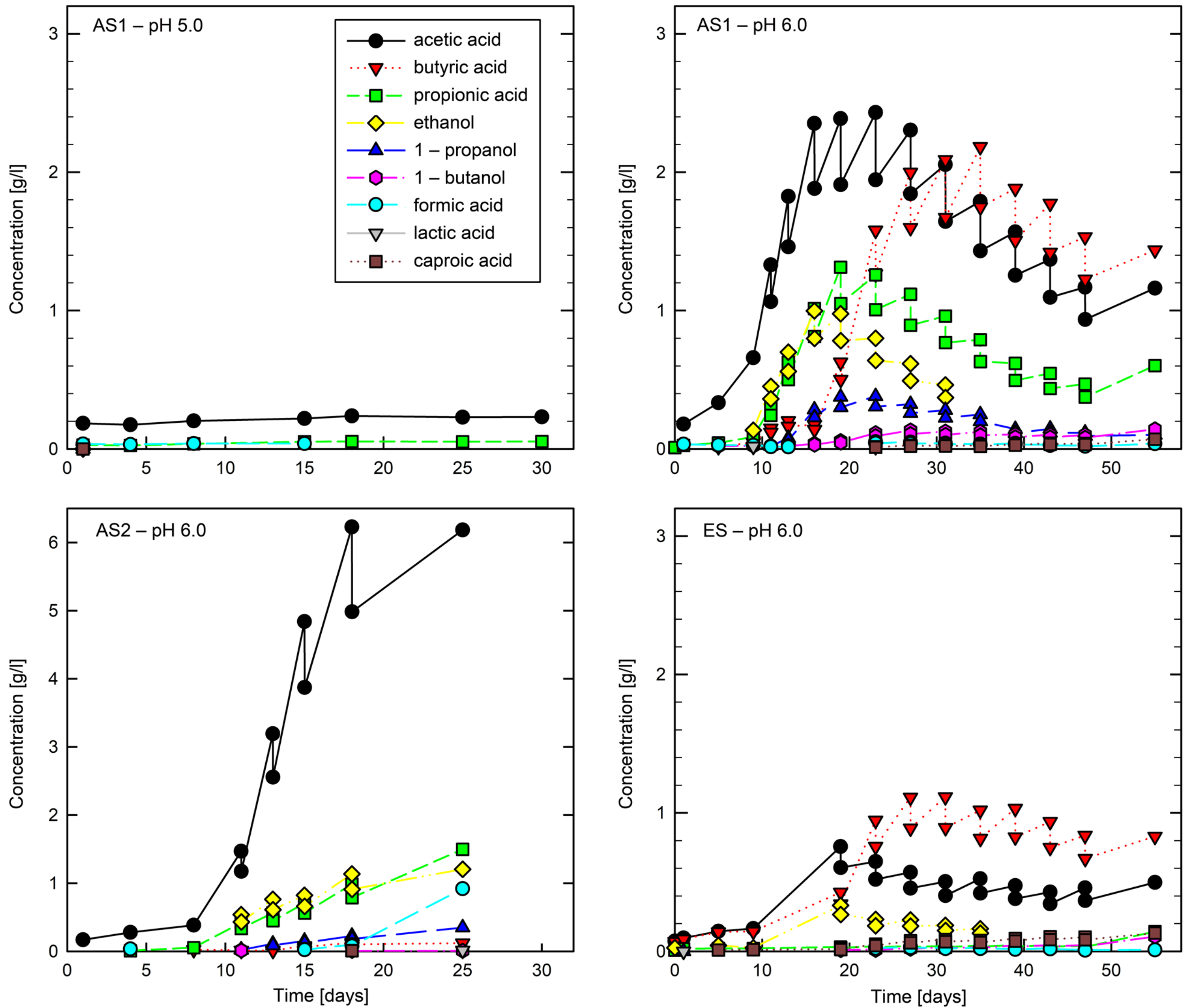

Fig. 5. Changes in metabolites concentration during repeated fed-batch dark fermentation of cellulose. Concentrations of metabolites before and after feeding are presented. Feeding days for each process: AS1 – pH 6.0 (11, 13, 16, 19, 23, 27, 31, 35, 39, 43, 47); AS2 – pH 6.0 (11, 13, 15, 18) ; ES – pH 6.0 (19, 23, 27, 31, 35, 39, 43, 47).

40 days. Then, a gradual decline in the hydrogen production rate was observed. This was accompanied by a substantial decrease in the amount of consumed cellulose and a lower concentration of the organic acids produced (Fig. 5). In the repeated fed-batch process butyrate was one of the main metabolites, while in batch experiments it was observed in low concentration. Stable hydrogen production in the repeated fed-batch process was also obtained for bacteria culture derived from the enrichment on starch (ES pH 6.0 – Fig. 4). However, this bacterial culture required more time to initiate hydrogen production and it was lower than for the AS1 at pH 6.0. Unfortunately, cessation of hydrogen production after about 40 days was observed for this process. Bacterial culture adapted previously to starch was, therefore, less effective in hydrogen production from cellulose. The highest yields were obtained for AS1 at pH 6.0 (Table IV). It was 1.04 mol

Table IV
Hydrogen yield in repeated fed-batch processes of hydrogen production from cellulose.

Inoculum	Hydrogen yield [mol H_2/mol$_{hexose}$] after			
	1 feeding cycle	2 feeding cycle	5 feeding cycle	9 feeding cycle
AS1 – pH 6.0	1.04	0.83	0.75	0.63
AS2 – pH 6.0	0.67	0.75	0.30	–
ES – pH 6.0	0.79	0.67	0.39	0.27

H_2/mol$_{hexose}$ (6.38 mmol H_2/g$_{cellulose}$) in the initial cycles. However, it decreased substantially during the process and dropped to 0.63 mol H_2/mol$_{hexose}$ after 40 days. In another study with sewage sludge and cellulose as a substrate maximum hydrogen yield reached 0.1 mol H_2/mol$_{hexose}$ during continuous operation at mesophilic conditions (Gadow et al. 2012). These results show that H_2 yields obtained from cellulose in long term processes are lower than those for batch studies. Nevertheless, obtained H_2 yields are promising and further research is needed to evaluate the long-term effect of inoculum pretreatment on direct hydrogen production from complex substrates.

Conclusions

The experimental results showed that pretreatment of the anaerobic sludge at 90°C was not sufficient to suppress H_2 consuming bacteria, both for starch and cellulose as the substrates. As a result, no hydrogen was observed at the end of batch experiments. However, anaerobic sludge was successfully enriched after pretreatment at 100°C on starch with a high and stable hydrogen yield of 1.7 mol H_2/mol$_{hexose}$. H_2 yield of culture enriched on cellulose reached 0.48 mol H_2/mol$_{hexose}$. Hydrogen consuming bacteria reduced H_2 production in a repeated batch process with cellulose because they regained their activity after several cycles. Results indicated that enrichment of mixed bacteria culture is highly substrate-dependent and enrichment on starch was more effective. Elimination of sulfate from medium resulted in 6.9 times higher an H_2 yield from cellulose, probably due to reduced activity of SRB. Hydrogen production in repeated fed-batch culture was effective in the initial stage of the process for AS1 at controlled pH 6.0, resulting in H_2 yield of 1.04 mol H_2/mol$_{hexose}$, but it became less efficient with time.

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Conflict of interest

The authors do not report any financial or personal connections with other persons or organizations, which might negatively affect the contents of this publication and/or claim authorship rights to this publication.

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