Chapter 5

Fermentative Hydrogen Production by Molasses; Effect of Hydraulic Retention Time, Organic Loading Rate and Microbial Dynamics

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http://dx.doi.org/10.5772/47750

1. Introduction

The generation of hydrogen by biological means is not energy intensive compared with the conventional thermochemical techniques, since the operating temperature and pressure are not very high. As raw materials organic waste streams can be used that can be considered as a renewable resource (Vijayaraghavan & Mohd Soom, 2006).The method of the dark fermentation has certain advantages compared with the other biological processes. In contrast to bio-photolysis and photo fermentation, the process needs no solar radiation, but the required energy is supplied by the organic substrates and hence the process is not interrupted during the night. Moreover, the production rate of the H_2 of the fermentative bacteria in comparison with the other biological processes is greater (Kumar et al., 2000; Nath et al., 2005).

The different process parameters that are relevant for hydrogen production have been surveyed (Li & Fang, 2007; Wang & Wan, 2009) and include the type of substrate, nutrient concentration, the inoculum, pH, reactor configuration, hydraulic retention time (HRT), organic loading rate (OLR). Carbohydrate-rich substrates are the most suitable for fermentative H_2 production systems (Hawkes et al., 2002; Kapdan & Kargi, 2006; Meherkotay & Das, 2008; Ueno et al., 2007) seeded with saccharoclastic microorganisms, They are able to break down organic substances via the Embden-Meyerhof pathway resulting to different metabolic products depending on the type of microorganism and the environmental conditions driving their catabolism (Hallenbeck, 2009).

The relevant microbial groups for the fermentative hydrogen production groups are clostridia and enterobacteria (Hallenbeck, 2005; Hawkes et al., 2007). Both groups were repeatedly experimentally confirmed as major hydrogen producers (Valdez-Vazquez & Poggi-Varaldo, 2009).

$C_6H_{12}O_6 + 2\,H_2O \rightarrow CH_3COOH + CH_3OH + 2\,CO_2 + 2\,H_2$	(1)
$C_6H_{12}O_6 + 2\,H_2O \rightarrow 2\,CH_3COOH + 2\,CO_2 + 4\,H_2$	(2)
$C_6H_{12}O_6 \rightarrow CH_3(CH_2)_2COOH + 2\,CO_2 + 2\,H_2$	(3)
$C_6H_{12}O_6 \rightarrow 0.6\,CH_3COOH + 0.7\,CH_3(CH_2)_2COOH + 2\,CO_2 + 2.6\,H_2$	(4)
$4\,H_2 + CO_2 \rightarrow CH_3COOH + 2\,H_2O$	(5)
$C_6H_{12}O_6 + 2\,H_2 \rightarrow 2\,CH_3CH_2COOH + 2\,H_2O$	(6)
$4\,H_2 + H_2SO_4 \rightarrow H_2S + 4\,H_2O$	(7)
$C_6H_{12}O_6 \rightarrow CH_3CH(OH)COOH + CH_3COOH + CO_2$	(8)
$C_6H_{12}O_6 \rightarrow 2\,CH_3CH(OH)COOH$	(9)
$3CH_3CH(OH)COOH \rightarrow 2\,CH_3CH_2COOH + CH_3COOH + CO_2 + H_2O$	(10)
$4\,CH_3OH + 2\,CO_2 \rightarrow 3\,CH_3COOH + 2H_2O$	(11)
$2\,CH_3OH + CH_3COOH \rightarrow CH_3(CH_2)_4COOH + 2\,H_2O$	(12)
$Fd_{red} + 2\,H^+ \rightarrow Fd_{ox} + H_2$	(13)
$CH_3CH(OH)COOH + 0.4\,CH_3COOH \rightarrow 0.7\,CH_3(CH_2)_2COOH + CO_2 + 0.4\,H_2O + 0.6\,H_2$	(14)

Table 1. Biochemical reactions relevant to hydrogen production

Enteric bacteria are gram-negative rods, facultative aerobic, with relatively simple nutrient requirements and can not form spores (Schmauder, 1992). Among the species that can produce H_2, are *Escherichia* (*E. coli*), *Proteus* (*P. vulgaris*), *Enterobacter* (*E. aerogenes*). Enteric bacteria ferment sugars to a variety of end products such as acetate, formate, lactate, succinate, ethanol, CO_2 and H_2. Hydrogen is produced according to equation 1 (Li & Fang, 2007). The maximum

possible hydrogen yield by this pathway is 2 mol H_2 per mol hexose. In experiments with intestinal bacteria rather half of this value has been found (Hallenbeck, 2005).

Clostridia are spore forming, gram-positive bacteria (Schmauder, 1992). Through sporulation they can survive for example dehydration, heat and large changes in pH. Clostridial catabolism includes a variety of reactions and hence fermentation end-products such as acetate, acetone (*C. pasteurianum*), butyrate (*C. butylicum*), butanol (*C. acetobutylicum*) or caproic acid (C*. kluyveri*) (Schmauder, 1992). Hydrogen, using hexose as a substrate can be produced by two pathways with acetate and butyrate as end-products as equations 1 and 2 describe (Hallenbeck, 2005; Hawkes et al., 2007; Li & Fang, 2007). That fact that clostridia can produce higher amount of hydrogen makes them more attractive and hydrogen systems aiming at their growth must be strived. In experiments with mixed cultures yields between 1.5 mol H_2/mol hexose and 2.5 mol H_2/mol hexose were achieved (Wang & Wan, 2009). In practice the highest yield is achieved when the catabolism is driven through a mixed acetate butyrate according to equation 4 (Lengeler, 1999). This is because hexose can be also metabolized by hydrogen neutral fermentation pathways with lactic acid, ethanol, acetone, butanol as end-products, or a portion of the substrate is consumed for the production of biomass, which theoretically can be 18.5% and 14.5% of the theoretical one through the acetate and butyrate hydrogen producing pathway respectively (Aceves-Lara et al., 2008). More over, a part of hydrogen that is already produced may be consumed by certain microorganisms as homoacetogens (equation 5) with acetate as end-product (Dworkin et al., 2006), or propionic acid bacteria (equation 6) (Li & Fang, 2007), or sulfate reducing bacteria (equation 7). The co-existence of microorganisms other than hydrogen producing that compete for substrate has been observed in many hydrogen producing systems (Hawkes et al., 2007; Hung et al., 2011a, 2011b; Li et al., 2011). A major part of them belongs to the lactic acid bacteria (LAB) distinguished to heterofermentative LAB, which produce lactic acid together with CO_2 (equation 8) and minor quantities of ethanol and acetic acid and the homofermentative LAB, which produce mainly lactic acid (equation 9) (Martinko & Clark , 2009). Through the monitoring of the metabolites of carbohydrate solely is not always possible to determine the metabolic pathays used by the bacteria, since many clostridia are capable of secondary fermentation. *C. propionicum* can for instance metabolize lactic acid for the production of propionate and acetate (equation 10), while some homoacetogens utilize ethanol and CO_2 yielding acetate (equation 11). In some cases, hydrogen can be also produced by secondary fermentation *C. kluyveri* can utilize ethanol and acetate yielding hexanoic acid and molecular hydrogen by the oxidation of reduced ferredoxin (Fd) (equation 12) and *C. tyrobutyricum* can transform lactate and ethanol to butyrate (equation 14) (Martinko & Clark, 2009). It is therefore obvious that biological hydrogen production systems are complicated in terms of biological processes and microbial species involved.

Aim of this work was to experimentally study the effect of HRT and OLR on bio-hydrogen production in terms of maximization of H_2 yield, so as to optimize substrate utilization efficiency that contributes to the cost effectiveness of the process. Experiments were carried out in large-lab scale reactors of 30 L working volume. In this way,

experience in the start-up procedure, selection of only H_2 producing microorganisms and the stability of long term continuous operation without methanogenesis of such a set-up could be gained. This can further be used for the scale-up of bio-hydrogen production towards the final aim of commercial implementation. An attempt towards the clarification of the possible metabolic pathways and the involved microorganisms, with along as their possible interactions was also undertaken. The way that these microorganisms behave and interract with each other and their milieu is very important for the design of effective hydrogen producing systems. The understanding of these processes can help the designer to manipulate hydrogen production by the suitable variation of the process parameters. With the use of molecular biological techniques it is possible to acquire better insights into such systems.

2. Materials and methods

2.1. Experimental set-up

Dark fermentation experiments were conducted in two identical 40 L reactors made of borosilicate glass (QVF) with a working volume of 30 L heated at 37 °C ± 2 °C by a heating pipe. The content of the fermenter was homogenized by external recirculation with eccentric screw pumps (Netsch). The pH was regulated by means of a pH glass electrode (Endress & Hauser, Orbisint CPS11) and a pH measuring transducer (Endress & Hauser, Mycom) connected to a programmable controller (Endress & Hauser, Memograph), which controlled 2 dosing pumps (Metrohm, Dosimat) for automatic addition of a sodium hydroxide solution 25% v/v and a hydrochloric acid solution 25% v/v, respectively. The Organic Loading Rate (OLR) was adjusted to the desired level by dosing (Metrohm, Dosimat) with molasses diluted 1:2 w/w and supplemented with nutrients. Every 100 mL of nutrient solution contained the following quantities in g; 1.72 $FeSO_4 \cdot 7H_2O$, 0.36 $CaCl_2 \cdot 2H_2O$, 3.78 KCl, 0.17 $MgCl_2 \cdot 6H_2O$, 11.46 NH_4Cl, 1.05 KH_2PO_4, 0.181 $FeCl_2 \cdot 4H_2O$, 0.041 $NiCl_2 \cdot 6H_20$, 0.021 $CoCl_2 \cdot 6H_2O$, 0.011 $ZnCl_2$, 0.170 KI, 0.177 $(NaPO_3)_6$, 0.0085 $MnCl_2 \cdot 4H_2O$, 0.0085 NH_4VO_3, 0.0085 $CuCl_2 \cdot 2H_2O$, 0.0061 $Al_2(SO_4)_3 \cdot 18H_2O$, 0.0085 $NaMoO_4 \cdot 2H_2O$, 0.0085 H_3BO_3, 0.0085 $Na_2WO_4 \cdot 2H_2O$, Na_2SeO_3 0.0085, 0.170 cysteine. Depending on the Hydraulic Retention Time (HRT) applied, the following quantities of this solution were added to the molasses solution; 33 mL for HRT > 2 d, 66 mL for 1 d< HRT < 2 d and 123 mL for HRT < 1 d.

The HRT was independent of substrate dosing. It was regulated by the pumping (Prominent, Gamma/L) of tap water and automatic removal of excess mixed liquor by a peristaltic pump (Ismatec, MPC Standard) controlled by a water lever sensor (Endress & Hauser, Liquiphant). The tab water was stored in containers and was daily sparged with N_2 in order to reduce the dissolved oxygen concentration bellow 1 mg/L. The produced biogas quantity was measured with a drum-type gas meter (Ritter, TG 05) and registered into the programmable controller. The produced gas was collected in gas bags (Lindte). In Figure 1 the experimental set-up is presented.

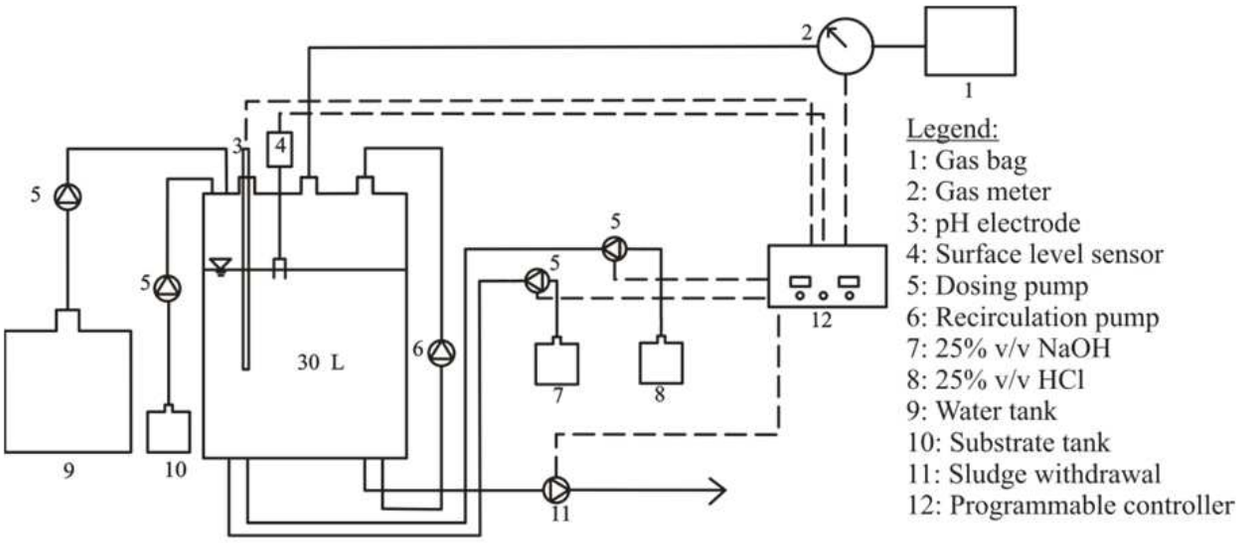

Figure 1. Experimental set-up

2.2. Reactor operation

The inoculum of the reactor has been acquired from the anaerobic digester of the Sewage Treatment Plant for Research and Education (LFKW) of the University of Stuttgart (Germany). It was diluted to 2% to 4% Total Solids (TS) concentration and sieved consecutively through 4 mm and 2 mm mesh size to prevent clogging of the tubing. It was then pretreated for 24 h at 105 °C, in order to kill the methanogenic bacteria. The dried sludge was pulverized and solved into tab water for the start-up of the system. As substrate sugar beet molasses acquired from a sugar factory in south Germany were used. In Table 2 the composition of molasses is presented. For the start-up of the system, the reactor was fed with 450 g of sucrose in a batch mode at pH 6.5 in order to enrich the biomass in H_2-producing microorganisms. Upon sucrose depletion continuous operation of the system was started. The pH was reduced to 5.5, a value that has been reported to be the optimum for continuous bio-hydrogen production (Mariakakis et al., 2011). The first phase of continuous operation aimed at the further selection of the biomass for hydrogen producing bacteria by application of high HRT and low OLR. The various experimental conditions tested during the continuous operation are presented in table 3. Their selection was based depending on the experimental progress as described in chapter 3.1. In many cases one of the two reactors had to be re-inoculated with seed sludge acquired by the other reactor. All phases had a minimum duration of 5 times the applied HRT. At phase DVII (table 3) Fe^{2+} at end-concentration in reactor of 1000 mg/L was added.

Molasses			
CODtot [mg/kg]	782000		
Ntot [mg/kg]	18610	Maltose [mg/L]	--
Ptot [mg/kg]	216	Acetate [mg/L]	1020
TS [g/kg]	848	Propionate [mg/L]	175
VS [%]	892	Butyrate [mg/L]	3062
Sucrose [g/kg]	520	D-Glucose [mg/L]	--
Lactose [mg/L]	--	Lactate [mg/L]	13736

Table 2. Composition of molasses

2.3. Analytical methods

The analyses of concern were determined according to the german standards (Deutsches Institut fuer Normung, 2002) and performed three times a week. These included; total solids (TS), volatile suspended solids (VSS), chemical oxygen demand (COD), a group parameter used for the detection of carbonaceous matter and nitrogen (in total and soluble form acquired after filtration through membrane with 0.45 μm pore diameter). Glucose, sucrose and lactic acid have been determined spectrophotometrically after enzymatic digestion by test kits according to the manufacturer's instructions (R-Biopharm). Gas Chromatography was used to analyze organic acids and alcohols. The sample was filtered through a 0.45 μm pore diameter filter and acidified with a 96% H_2SO_4 solution. Organic acids, ethanol and butanol were detected by GC (Perkin Elmer) mounted with a fused silica capillary (Varian) and using a flame ionization detector. Both the injection and capillary temperatures were set at 280 °C. Biogas composition was determined once daily after up-grading for particulate matter and water vapor removal by a gas analyzer (ABB, AO2020), equipped with an infrared detector for CH_4 and CO_2 and a thermal conductivity detector for H_2.

DNA extraction, 16S rDNA of eubacteria and clostridia PCR amplification, DGGE analysis and sequencing were performed as previously described (Mariakakis et al., 2011).

3. Results and discussion

3.1. Reactor operation

In figure 2, the reactor operation parameters, gas production and hydrogen yield performance for the experimental phases DI to DIII are presented.

After the start-up of the reactor for a week, the HRT and OLR were reduced to 4 d and 11.6 g sucrose / (L·d) respectively. After about 15 d a continuous H_2 production could be established (phase DI), which stabilized after approximately 30 d. The average soluble COD concentration in the reactor was app. 60 g/L and the average H_2 yield 2.47 mol H_2/mol hexose.

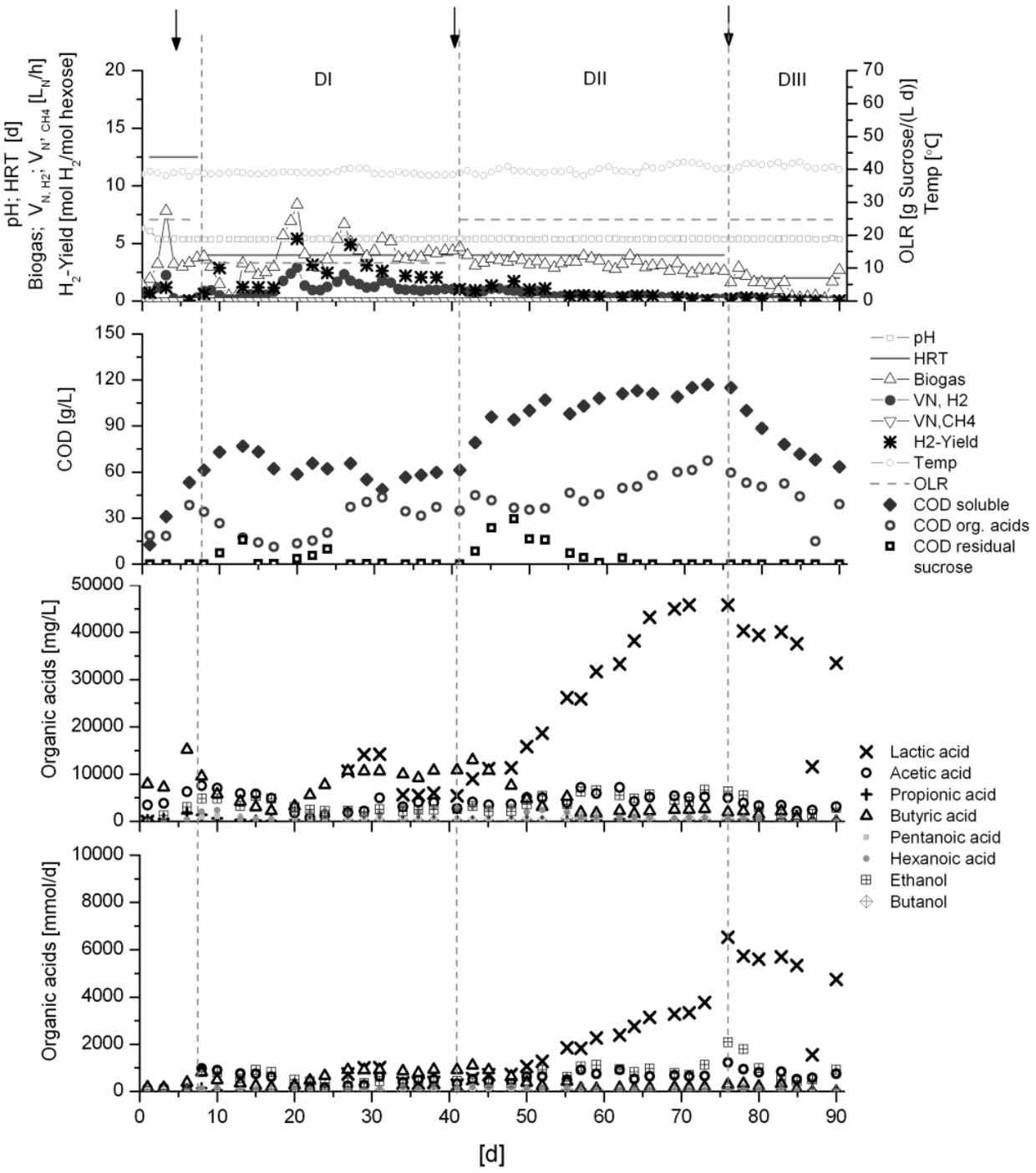

Figure 2. Operation parameters, gas production, hydrogen yield performance and production rate of relevant metabolites during phases DI to DIII. Arrows indicate the sampling dates for microbial population analyses

Phase		DI	DII	DIII	DV	DVI	DVII	D1	D2	D3	D4	D5	D6	D7
OLR [g sucrose/(L d)]		11.6	24.7	24.7	45.9	36.1	36.1	11.6	24.7	24.7	36.1	45.9	36.1	69.6
HRT [d]		4.0	4.0	2.0	1.0	2.0	0.5	3.0	3.0	1.0	1.0	0.5	0.5	0.3
Duration [xHRT]		8	11	7	20	10	47	5	6	7	20	40	41	120
Biogas [L_N/h]		3.93	3.31	1.33	3.47	6.72	8.39	2.23	2.31	2.32	9.72	6.66	6.58	5.57
H_2	[%]	32	18	8	20	16	31	22	25	18	26	26	32	35
	[L_N/h]	1.11	0.53	0.11	0.61	1.07	2.42	0.44	0.65	0.49	2.19	1.51	1.82	1.68
COD soluble [g/L]		63	101	84	61	98	27	45	97	43	49	34	22	20
Biomass	[g/L]	3.3	2.5	4.5	2.1	2.2	3.7	1.3	1.9	2.0	3.0	1.6	1.6	1.3
	[g/d]	25	19	66	64	34	161	13	19	59	89	92	94	147
Sub/te degradation [%]		93.9	92.3	100.0	92.4	100.0	99.4	99.9	71.5	100.0	99.4	95.9	97.2	83.1
Yield [mol H_2/mol hexose]		2.49	0.68	0.12	0.43	0.63	1.68	1.00	0.38	0.91	1.53	0.97	1.31	0.71
HLa	[mg/L]	9365	20252	35467	25577	36767	787	9989	13024	12309	2325	5045	2422	1451
	[mmol/d]	647	1949	5029	7447	5361	349	872	1168	3497	556	2715	1230	1276
HAc	[mg/L]	3962	4904	3334	6848	3745	4446	2581	6260	2758	5619	6183	4159	2425
	[mmol/d]	506	619	814	3495	944	4215	478	1053	1364	2788	6172	4149	4571
HPr	[mg/L]	251	82	131	41	249	0	47	31	416	277	40	0	0
	[mmol/d]	25	7	25	15	49	0	3	4	167	109	31	0	0
HBu	[mg/L]	7390	4861	1230	3678	9828	5877	4949	3683	3275	11927	5451	4064	2752
	[mmol/d]	634	393	184	1221	1674	3796	594	515	1078	3998	3646	2761	3461
HVa	[mg/L]	312	60	95	0	137	0	51	14	132	69	0	0	0
	[mmol/d]	24	5	14	0	21	0	5	1	39	20	0	0	0
HCa	[mg/L]	1805	1660	349	0	2104	30	2243	2023	131	0	0	439	44
	[mmol/d]	110	111	45	0	279	15	197	177	34	0	0	38	43
EtOH	[mg/L]	3217	4492	3194	282	1514	0	1283	1934	1468	663	194	643	0
	[mmol/d]	547	764	1042	190	513	0	287	433	963	435	255	845	0
BuOH	[mg/L]	74	281	177	0	49	0	1149	135	5	0	0	0	0
	[mmol/d]	4	30	36	0	10	0	159	19	2	0	0	0	0

Table 3. Experimental phases, operating parameters and average concentrations and yields of produced gases and metabolites

On the 41st day of operation the OLR was increased from 11.6 g sucrose / (L·d) to 24.7 g sucrose / (L·d) (phase DII), which caused an increase to the residual sucrose concentration in the reactor during the first days. The biomass was not able to metabolize the total amount of substrate. Over the total 35 d of operation of this phase H_2 in the produced biogas gradually decreased and after 70 d no H_2 was detected. The fact that biogas production was sustained, indicated biological activity, but not towards H_2 production. In phase DIII the HRT was reduced to 2 d with the OLR retained unchanged. The concentration of soluble COD decreased gradually from 100 g/L to 60 g/L due to the higher dilution rate. Nevertheless, H_2 production could not be restored and the reactor operation was terminated.

After one month of operation the excess sludge from reactor a (R-a) was used to inoculate reactor b (R-b) (phases D1 to D7). In figure 3 the reactor operation parameters, gas production and hydrogen yield performance for the experimental phases D1 to D7 together with the data from phase DI for continuity and comparison reasons are presented. Biogas and hydrogen production started immediately upon seeding of the reactor at HRT of 3 d and OLR of 11.6 g sucrose / (L·d). Hydrogen production was stable, but the yield was lower than 1 mol H_2/mol hexose. The concentration of soluble COD did not exceed 45 g/L. Increase of the OLR at 24.7 g sucrose / (L·d) lead to steep increase of the soluble COD and COD-sucrose concentration above 120 g/L and 60 g/L respectively and a decrease of the COD concentration of the metabolites. It seems that in the beginning the biomass concentration was not sufficient to metabolize the whole amount of sucrose, which on its turn accumulated in inhibitory levels as demonstrated by the cease of biogas and hydrogen production and the reduction of the COD-organic acids. It has been reported (Hafez et al., 2010) that glucose can become inhibiting for residual concentrations above 20 g/L. This problem could be overcome by the reduction of HRT to 1 d (phases D3 and on). H_2 production was restored immediately and was maintained until the termination of the experiment due to time constraints. Biogas and hydrogen production exhibited fluctuations over time. These can be generally attributed to the experimental procedure followed, which affected the actual HRT and OLR. The dosing of water and substrate was stopped everyday for different periods each time, in order to be prepared as described in Materials and methods. Stronger fluctuations were related mostly to the failure of substrate dosing due to tube clogging. In all phases, except phases D4 and D6, the H_2 yield was equal or lower than 1 mol H_2/mol hexose (table 3). In phase D4 it reached 1.53 mol H_2/mol hexose for HRT of 1 d and OLR of 36.1 g sucrose / (L·d) and in phase D6 1.31 mol H_2/mol hexose for for HRT of 0.5 d and OLR of 36.1 g sucrose / (L·d).

During the whole R-b operation, which together with the time period of phase DI reached 180 d, no methane was detected. Methanogenesis could be inhibited through the thermal pre-treatment of the seed sludge and the selected operation parameters were sufficient for hindering the proliferation of the methanogens in the system. In our previous work for which no pre-treatment of the seed sludge was carried out methanogenesis could be only be inhibited for 120 d (Mariakakis et al., 2011). Sucrose in higher concentrations could be detected only in phases D2 and D7 resulting in average substrate degradations of 71.5% and 83.1% respectively. In D2 sucrose accumulated in the beginning of the phase as results of the long HRT and the OLR of 24.7 g sucrose / (L·d). At D7 sucrose could not be degraded due to the high OLR of 69.6 g sucrose / (L·d) and the short HRT of 0.25 d.

The excess sludge from phase D3 of R-b was used to re-inoculate R-a (phases DV and DVI). In phase DVII the reactor was inoculated with excess sludge of phase D6. In Figure 4 the reactor operation parameters, gas production and hydrogen yield performance for these experimental phases together with the data from phase D3 for continuity and comparison reasons are presented.

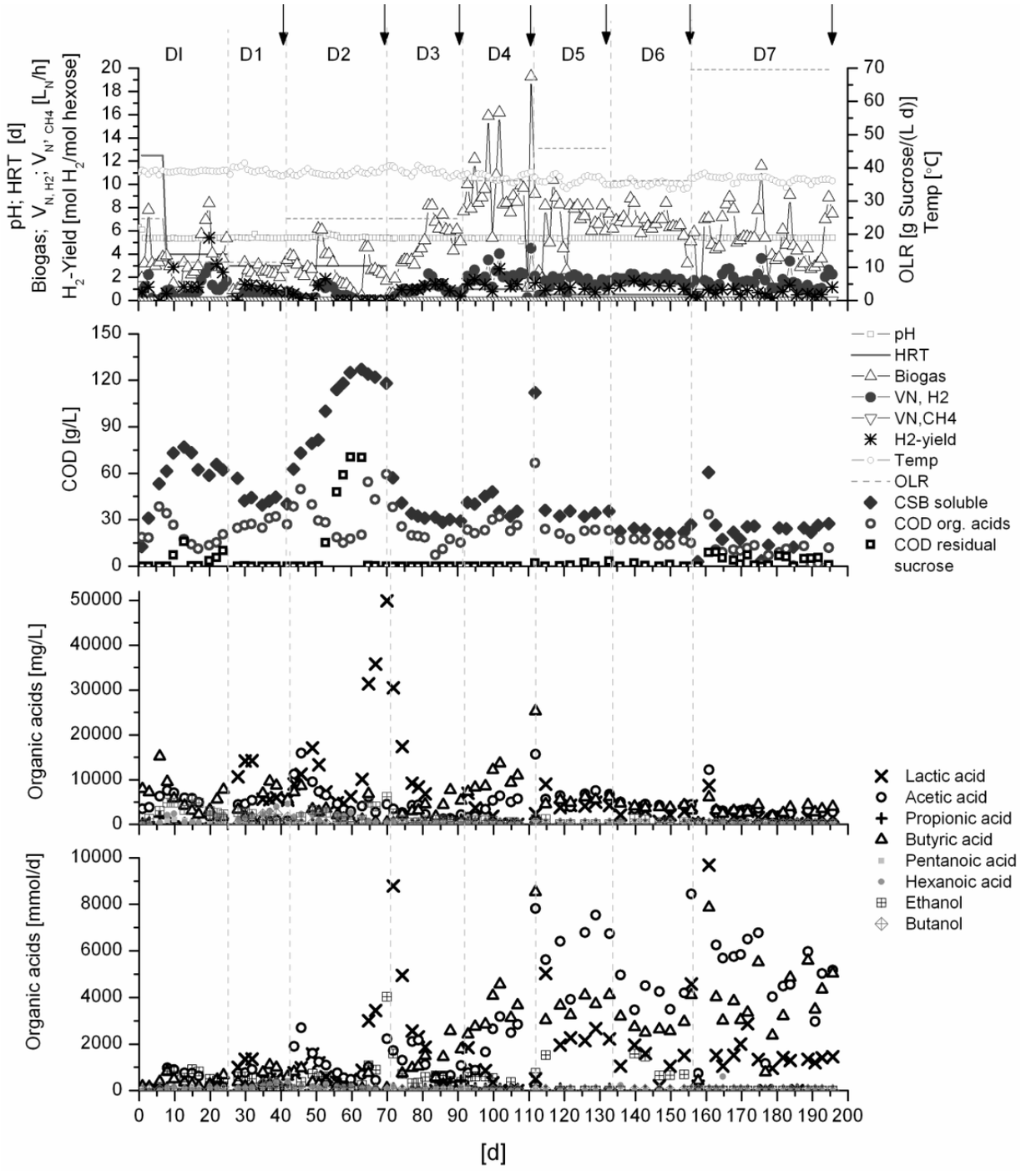

Figure 3. Operation parameters, gas production, hydrogen yield performance, HBu:HAc ratio and production rate of relevant metabolites during phases DI to D7. Arrows indicate the sampling dates for microbial population analyses

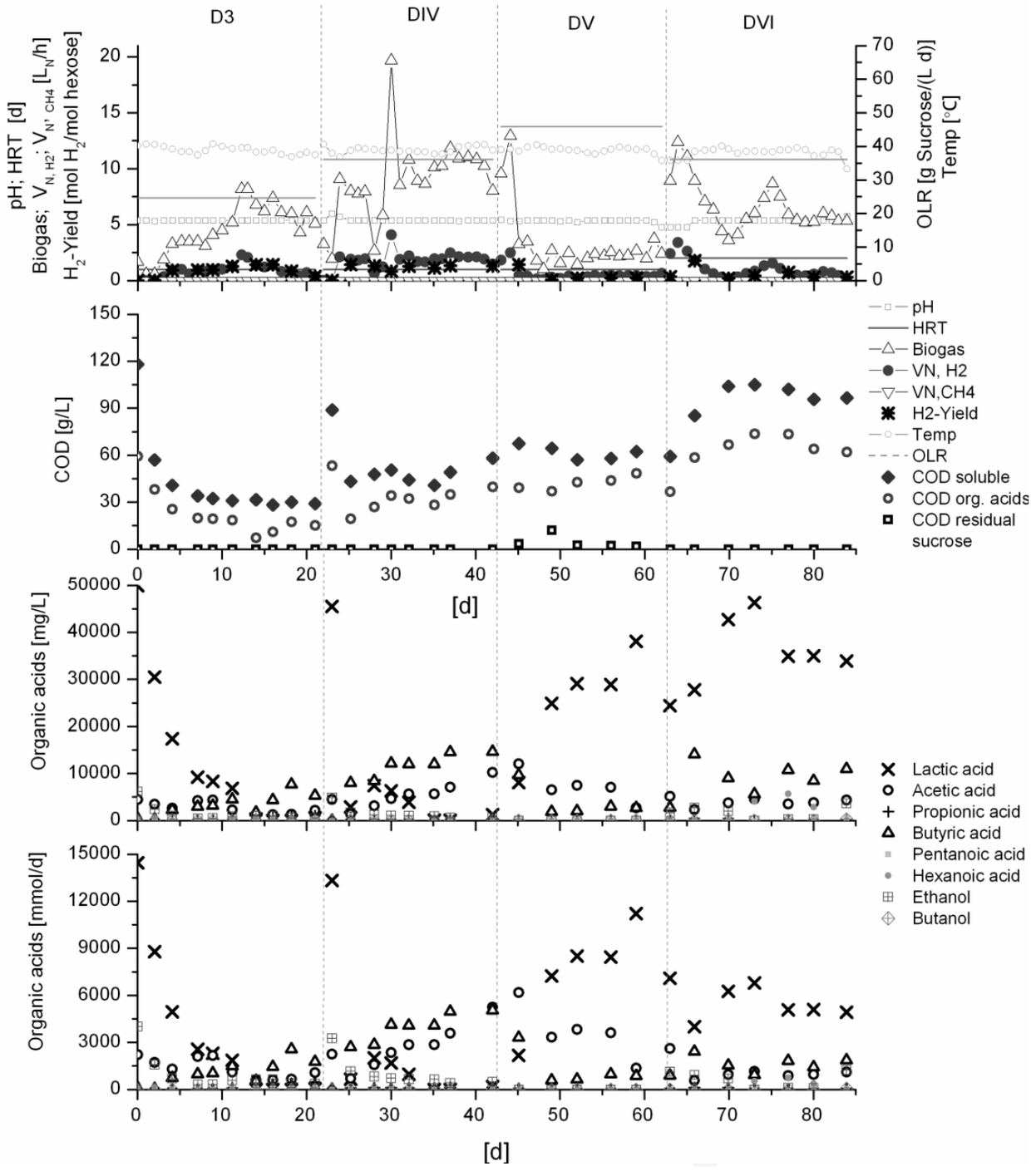

Figure 4. Operation parameters, gas production, hydrogen yield performance, HBu:HAc ratio and production rate of relevant metabolites during phases D3 to DVII. Arrows indicate the sampling dates for microbial population analyses

Biogas and hydrogen production started also in these cases immediately after seeding of the reactor. Due to the OLR set at 47.1 g sucrose / (L·d), the average soluble COD concentration reached 61 g/L. H_2 production (phase DV) yieldied 0.43 mol H_2/mol hexose. The decrease of the OLR to 36.1 g sucrose / (L·d) and increase of HRT to 2 d in phase lead to further increase of the soluble COD to an average concentration of 98 g/L. Hydrogen yield was slightly

improved and reached 0.63 mol H_2/mol hexose. Biogas and hydrogen production during phase DVII were instable mainly due to malfunctions of the substrate dosing. The hydrogen yield for addition of Fe^{2+} achieved was 1.68 mol H_2 / mol hexose, which corresponds to an increase of approximately 28 % in comparison to phase D6.

The addition of Fe^{2+} enhanced hydrogen production. From table 3 it can be seen that biomass production rate reached the highest value of 161 g/d comparable only with that of phase D7 that was acquired for double as much OLR. For comparison at phase D6, biomass production rate was only 94 g/d. It seems that this nutrient that is important for H_2 production as will be explained in chapter 3.2 was limiting for the biomass growth, since it was not included in high enough concentrations in the nutrient solution with which the molasses solution was supplemented.

In Table 4 the hydrogen yield of other works in comparison to the best acquired by this work are presented. In most of the cases slightly better results were acquired by the other researchers. In a semi-scale reactor operated at HRT of 0.25 d and pH 4.5 hydrogen yield up to 1.86 mol H_2/mol hexose was achieved (Ren et al., 2006). Lay et al. (2010) maximized the yield for HRT of 0.5 d and pH of 5.5 like in this work, but achieved a somewhat lower yield of 1.35 mol H_2 / mol hexose, even though the reactor set-up was considerably smaller permitting for better control of operation. Aceves-Lara et al (2008) operated a 2 2 L CSTR at HRT of 0.25 d and pH of 5.5. The maximum yield acquired was 1.70 mol H_2 / mol hexose for an OLR of 24.2 g COD / (L·d). For OLR as high as 77.2 g COD / (L·d) the acquired hydrogen yield was much higher than that of the current work (0.84 mol H_2 / mol hexose) for the same HRT and OLR of 69.6 g sucrose/(L·d), which is equivalent to 107 g COD / (L·d) (Aceves-Lara et al., 2008). It is obvious that the process can be further optimized.

Work		OLR [g COD/ (L d)]	HRT [d]	pH	$V_{Reactor}$ [L]	Duration [d]	H_2-Yield [mol H_2/ mol Hexose]	Degradation [%]
Ren et al. (2006)		6.32	0.44	4.5	1480	10	0.34	--
		27.98	0.25			10	1.86	--
		42	0.162			10	1.81	--
Lay et al. (2010)		40	1	5.5	4	130	0.48	60.2
		80	0.5			130	1.35	64.3
		120	0.67			130	0.89	68.1
Aceves-Lara (2008)		24.2	0.25	5.5	2	17	1.70	100
		48.5	0.25			12	1.62	100
		77.2	0.25			18	1.26	98
Own	(D4)	50 (36.1)	1	5.5	30	20	1.51	99.4
	(D6)	50 (36.1)	0.5			21	1.31	97.2
	(DVII)	50 (36.1)	0.5			25	1.68	99.4

Table 4. Operating parameters, efficiencies and hydrogen yields of works with molasses as substrate for fermentative hydrogen production

3.2. Microbial metabolism

From Figures 2 to 4 and Table 3 it can be seen that the major metabolites for all tested parameters were lactic, acetic, and butyric acid together with ethanol. Propionic and hexanoic acid were produced in low quantities and only in same cases. Pentanoic acid and butanol were not detected at all. Acetate and butyrate are typical for H_2 production by mixed acid fermentation of sucrose as already mentioned in introduction. Lactate can be produced by either lactic acid bacteria or by bacteria of the genus *Clostridium* via a pathway that does not promote H_2 production (Hiligsmann et al., 2011).

During phase DI lactate was produced in less quantity than acetate and butyrate with the later being produced in almost the same proportion indicating a mixed acid fermentation, even though the theoretical yield of 2.6 mol H_2 / mol hexose was not reached (equation 4). The increase of the OLR in phases DII and DIII has lead to gradually increasing lactate production rate with simultaneous increase of acetate into a lesser extent and decrease of butyrate. The higher substrate availability forced the biomass to shift its metabolism from mixed acid fermentation to lactate fermentation. The fact that biogas production also declined is an indicator for homolactic fermentation by LAB, during which only lactate is produced (equation 9). In phases DV and DVI lactic acid production could not be solely due to homolactic fermentation, since biogas was produced. Higher lactic acid production than acetic and butyric acid production was observed in the phases for which HRT and OLR were equal or higher than 1 d and 24.7 g sucrose / (L·d) respectively. The exact mechanism of lactic acid production can be explained either by the co-existence of LAB (Hafez et al., 2009) or by the metabolic shift of the hydrogen producing clostridia (Minton & Clarke, 1989). In all cases though, high lactic acid production was combined to an increase of the OLR (Oh et al. 2004; Kim et al., 2006; Oh et al., 2004; Hafez et al., 2009) and caused a diminution of hydrogen yield. The effect of lactic acid as a metabolite in hydrogen systems has not yet been clarified. It has been reported to be promoting to hydrogen production at low concentrations and inhibiting at high concentrations. In a work (Baghchehsaraee et al., 2009), an increase of the hydrogen yield combined with the complete degradation of the externally added lactic acid in concentrations up to 3 g/L was observed. In another work, Kim et al. (2012) also observed an increase of 22% in hydrogen yield when lactic acid up to 8 g/L was added to batch fermentors operated at pH of 4.5, and a reduction when the concentration was raised at 18 g/L. The corresponding undissociated form of the lactic acid, which is the potential inhibitor (van Ginkel & Logan, 2005) was 21 mmol/L and 45 mmol/L at pH 4.5 according equation 15. In this work, the highest lactic acid concentration was 35 g/L (9 mmol/L undissociated lactic acid at pH 5.5) and was only reached temporarily in the beginning of phase D3 without any obvious long-term negative influence on the hydrogen process, like in phase DIII. It seems that lactic acid was not the inhibition factor, but another substance that was not monitored.

$$pH = pK_a + \log\frac{A^-}{HA} \tag{15}$$

For phases DI to DIII and D1 to D3 during which OLR lower or equal to 24.7 g sucrose / (L·d) was applied, acetate and ethanol were produced in almost the same rates. A ratio of EtOH:HAc equal to 1:1 has also been proposed for clostridia as described by equation 1 for enteric bacteria, when hydrogen is evolved only through the conversion of Acetyl-CoA to pyruvate, yielding 2 mol H_2 / mol hexose (Minton & Clarke, 1989). For higher loadings the observed ethanol production rate diminishes either due to a metabolic shift of the biomass or due to its consumption. Ethanol has also been detected in other hydrogen producing systems operated at pH 5.5 and various HRT and OLR (Gavala et al., 2006; Karadag & Puhakka, 2010a; Kim et al., 2006; Shen et al., 2009) as a product either of enterobacterial (Hallenbeck, 2005), clostridial hydrogen production (Akutsu et al., 2009; Lin & Lay, 2004), or heterolactic bacteria (Kandler, 1983). In the case of clostridia, it has been suggested that ethanol is produced during the late growth phase during which no hydrogen is produced, while H_2 production is favored during the exponential growth phase, during which the organic acids are produced (Nath & Das, 2004), yielding at the end of a batch fermentation a ratio of 1:1. By the adjustment of short HRT in CSTR it is possible to maintain the bacterial population in the exponential growth phase. However, the adjustment has to be suitable so that the biomass concentration in the reactor can be also maintained in concentrations that are suitable for high substrate conversion rates.

Propionic acid was produced in phases D3 and D4. Sucrose fermentation to propionic acid is a sink for hydrogen and according to equation 6 for each mol of propionic acid produced 1 mol of hydrogen is consumed. The derived hydrogen consumptions for phases D3 and D4 correspond to 15% and 19% of the total produced hydrogen respectively.

Hexanoic acid was mainly produced during phases DI, DII, DVI and D2. The HRT of all these phases was equal to or longer than 2 d. The production of hexanoic acid can only be explained by a possible secondary fermentation of *C. kluyveri* as described by equation 12 and 15.

The addition of Fe^{2+} in phase DVII did not influence the production rates of acetic acid in comparison to phase D6. On the other hand, the production rate of lactic acid was significantly reduced from 1230 mmol/d to 329 mmol/d corresponding to approximately 72% and ethanol was not produced anymore. Parallel, the production rate of butyrate increased approximately by 27%. The overall bacterial metabolism was shifted to butyrate fermentation and biomass growth as described in 3.1. This is an indication that lactic acid and ethanol production in the phases with relative short HRT (<1 d) was mainly due to the clostridial metabolism. They contributed more than 2/3 to the total lactic acid production. Iron is very important to hydrogen production, which is produced when the simple reaction of Eq. 16 takes place. This reaction is catalyzed in clostridia by a dimetallic iron only [FeFe]-hydrogenase, which receives protons by the reduced form either of ferredoxin or of NADH (Vignais & Billoud, 2007). Under iron limitation the activity of hydrogenase is also limited (Valdez-Vazquez & Poggi-Varaldo, 2009), pyruvate can not be degraded through the pathways leading to hydrogen, but fermentation is shifted towards lactic acid production (Minton & Clarke, 1989).

$$2\ H^+ + e^- \leftrightarrow H_2 \tag{16}$$

3.3. Microbial population

3.3.1. Species description and influence of HRT and OLR

The sample times for the phylogenetic analysis and the affiliated dominant species present in each experimental phase are indicated in figures 2 to 4. In figures 5 and 6 the PCR-DGGE profiles of the *Eubacteria* and *Clostridium* species are presented. The investigation on eubacterial 16S rDNA (table 5) showed mostly lactic acid bacteria of the Phylum *Firmicutes* as closest relatives to the detected DGGE bands. The analysis of 16S rDNA for clostridia showed repeating DGGE patterns (figure 5). In many cases several single bands in one lane showed the same sequence and could be affiliated to one *Clostridium* species, though it was possible to allocate several bands to different clostridia species. Most of the bands could be affiliated to the species *C. butyricum*, *C. tyrobutyricum* and *C. ljungdahlii* (table 5).

Carnobacterium sp. AT12 was the only *Eubacterium* species found in the seed sludge after the thermal pre-treatment and before start-up (table 6). It is a non-spore forming, facultative anaerobic and heterofermentative lactic acid bacterium (de Vos, 2009), which previously belonged to the genus of *Lactobacillus* (Dworkin et al., 2006). Even though this bacterium is no spore forming, it was able to survive the thermal pre-treatment. After the addition of substrate it was replaced by other species of LAB. The members of this genus are facultative anaerobic, mesophilic, non-spore forming, obligately saccharoclastic with complex nutritional requirements. Their optimum pH for growth is between 5.5 and 6.2, but they can also grow for pH lower than 5. *L. fermentum* is obligately heterofermantative. It degrades hexose to equimolar quantities of lactic acid, CO_2 and acetate or ethanol via the 6-phosphogluconate pathway.

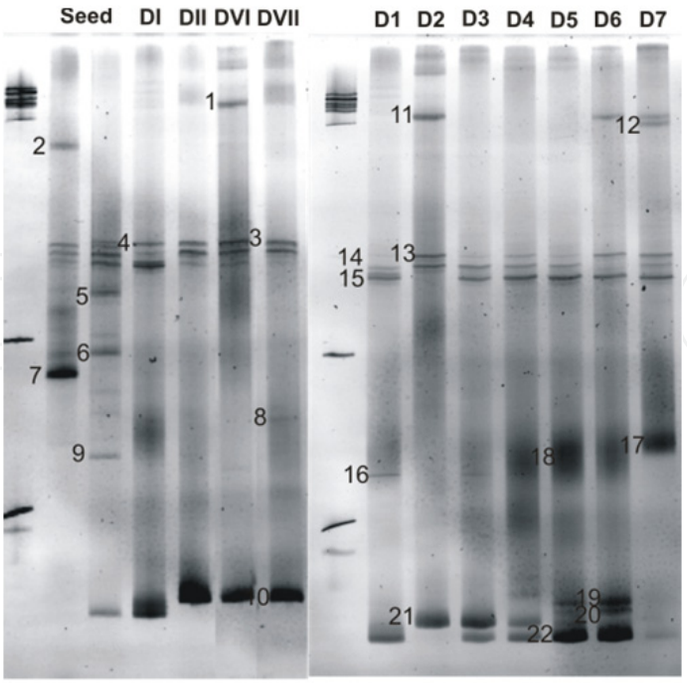

Figure 5. DGGE-profile of the *Eubacterium* species from each experimental phase. The numbers denote the bands that have been sequenced and successfully allocated. The sequencing results are presented in Table 5

L. delbrueckii subsp. *bulgaricus* on the other hand is obligately homofermentative and degrades hexose via the Embden-Meyerhoff pathway to lactic acid (de Vos, 2009). Through the phases DI to DII species of *Lactobacillus* established a stable community in the reactor. At phases DI and DII *Olsenella* species were also detected, while at phase DIII *Sporolactobacillus* has been identified (table 6). Species of the genus *Sporolactibacillus* are facultative anaerobic and obligately homofermentative. They can form spores, which are resistant to heating at 80 °C for 10 min (de Vos, 2009). They can grow only on fermentable carbohydrates at pH greater than 4.5. Their optimum growth temperature is 30 °C (Dworkin et al., 2006). For *Olsenella* sp. oral taxon 809 there are no metabolic data available, since it has not been cultivated. In general the species genus *Olsenella* is non-spore forming, homofermentative and strictly anaerobic. The end-products of sucrose fermentation consist mainly of lactic acid and to a lesser extent of acetic acid (Olsen et al., 1991). The presence of these bacteria can explain the increasing production rate of lactate and acetate indicating an overall shift of the bacterial metabolism in the system to lactic acid fermentation and more specific to homolactic fermentation as derived by the decreasing biogas production. They compete with the hydrogen producing bacteria for substrate.

In the seed sludge three species of *Clostridium* could be identified. Only *C. butyricum* could be maintained in the system until the end of phase DIII (table 6). The rest of the initially present *Clostridium* species were replaced by others indicating a constant changing population, like in the case of (Huang et al., 2010). All of them are able to produce copious amounts of hydrogen gas by mixed acid fermentation with acetate and butyrate as their major end-products as already mentioned. Even though, hydrogen producing bacteria where identified during phases DI to DII, the deterioration of hydrogen production could not be hindered. The lactic acid bacteria could proliferate in the system.

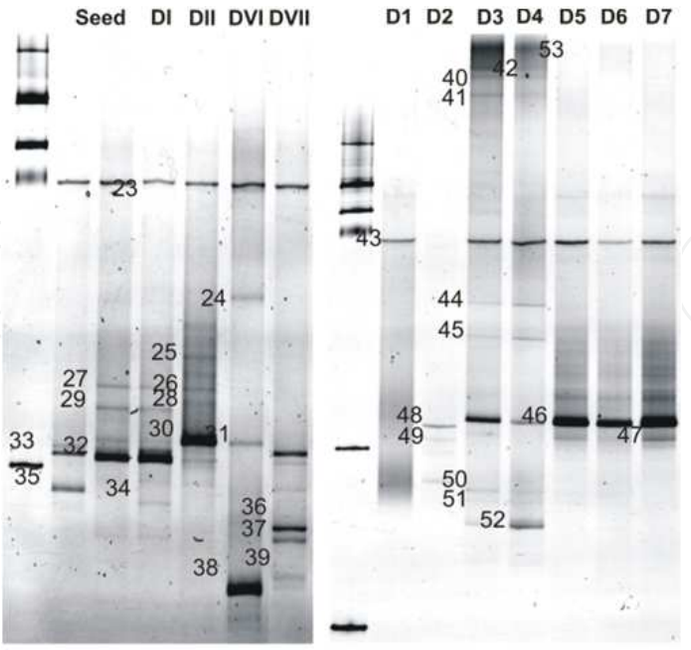

Figure 6. DGGE-profile of the *Clostridium* species from each experimental phase. The numbers denote the bands that have been sequenced and successfully allocated. The sequencing results are presented in table 5

Also in the case of phases DVI, D1 and D2 (tables 6 and 7) the symbiosis of lactic acid together with hydrogen producing bacteria could be observed resulting to high lactic acid production rates and medium to low hydrogen yields. In all cases, the employed HRT was equal or higher to 2 d. It seems that long HRT contribute to the dominance of lactic acid over hydrogen producing bacteria. In the case of phases D3 to D7, high production rates of lactic acid could still be observed, but they were lower than that of acetic and butyric acid, while medium to higher hydrogen yields could be achieved. The HRT applied was equal to or lower than 1 d. Under these conditions the hydrogen producing metabolism could dominate over the lactic acid metabolism. After phase D3 and on *C. tyrobutyricum* became one of the dominant *Clostridium* species. After phase D5 and on *Sporolactobacillus* could not be found any more in the system. It seems that HRT lower than 0.5 d does not support its growth. In the work of Fang (2002) who investigated hydrogen production by untreated secondary sludge at pH 5.5, loading rate of 25 g sucrose / (L · d), HRT 0.25 d and temperature of 26 °C, 69.1% of the microbial clones were affiliated to *Clostridium* species and 13.5% to *Sporolactobacillus racemicus*, which was able to be retained in the system due to the granular sludge reactor configuration.

Analogously, *Olsenella* disappeared from the system at phase D7 for HRT of 0.25 d. Lo et al (2008) could detect *Olsenella* and *C. butyricum* in a hydrogen producing reactor with xylose as substrate at HRT of 2 hours. They also used granular sludge. In another work (Castelló et al., 2009), the acquired low hydrogen yield was justified by the presence of *Olsenella* along with *Prevotella*, *Bulleidia*, *Mitsoukella* and *Selonomonas* species, which consumed the substrate. In this work, *C. tyrobutyricum* became visible also through the eubacteria specific primers indicating a general proliferation of *Clostridium* species for vey short HRT that comes in agreement with the low doubling times of 30 min to 3 h that have been observed for most *Clostridium* species (Dworkin et al., 2006). Despite the dominance of hydrogen producing biomass over lactic acid bacteria, the hydrogen yield was relatively low due to the low sucrose degradation efficiency. The biomass concentration in the system was not sufficient to metabolize the whole amount of substrate. *L. mucosae*, an obligate heterolactic bacterium, could maintain itself in the system even for HRT as low as 0.25 d. These findings are in agreement with the observations of (Kim et al., 2012), who investigated hydrogen production by a lactate-type fermentation in a CSTR with working volume of 4 L, glucose as substrate and digester sludge as inoculum pre-treated with acid. For HRT of 1 d and OLR equal to 10 g / (L·d) they affiliated 65% and 35% of the total bacterial population to *Eubacterium* and *Clostridium* species respectively. When the OLR was increased to 40 g / (L·d) the proportion changed in favor to *Eubacterium* species to 72% and 28% respectively. For the same OLR but HRT equal to 0.5 d a balance between the two populations with 50% each was established.

C. ljungdahlii could be detected in the system during the phases DIV, DVI, DVII and D1 to D7. More than 90% of its strains can produce H_2 during heterotrophic growth on glucose or fructose, while sucrose can not be utilized (de Vos, 2009). It can also grow autotrophically on H_2 and CO_2 or CO. It forms spores only rarely. The regulation of the diverse pathways of homoacetogens is still not understood (Dworkin et al., 2006). For the first case, extracellular enzymes released to the bulk liquid by other microorganisms that are able to degrade sucrose to glucose and fructose are required. In a mixed acid fermentation as described by

Eq. 4, a ratio of HBu:HAc should have been obtained. In most of the cases this ratio is lower than one indicating that acetic acid is also produced through a pathway other than that of hydrogen production. It seems that *C. ljungdahlii* is consuming hydrogen.

Band No.	**Affiliation**	**Similarity**	**Accession Number**
1	*Lactobacillus mucosae* strain TB-H32	97	AB425938
2	*Carnobacterium* sp. AT12	91	DQ027062
3	*Lactobacillus mucosae* strain FSL-04 1	87	JN092131
4	*Lactobacillus delbrueckii* subsp. *bulgaricus* strain CH3	85	JN675227
16; 18; 19; 20; 22	*Olsenella* sp. oral taxon 809	84-95	GU470903
5	*Lactobacillus fermentum* strain -O rkz-4	90	JN836490
6	*Lactobacillus delbrueckii* subsp. *bulgaricus* 2038	98	CP000156
7	*Carnobacterium* sp. AT12	93	DQ027062
8	*Lactobacillus casei* strain GIMC8:TVS-72	91	JF728260
9	Uncultured *Burkholderiales* bacterium clone CA38	96	EF434370
10; 21	*Sporolactobacillus* sp. MB-051	90; 91	AB548940
11	*Lactobacillus mucosae* strain LAB87	95	EF120376
12	*Clostridium tyrobutyricum* 5S	88	L08062
13	*Lactobacillus mucosae* strain SF1031	94	FN400925
14	Marine bacterium strain SJ-BF7	84	AM260710
17	*Clostridium tyrobutyricum* strain SCTB132	94	JN650297
23; 29; 32	*Clostridium butyricum* strain CB TO-A	94-100	AB687551
24; 39; 43; 44; 45; 50; 51; 52	*Clostridium ljungdahlii* type strain DSM13528T	87-100	FR733688
25; 26; 28; 30; 31; 40; 41; 42; 46; 47	*Clostridium tyrobutyricum* strain SCTB130	80-100	JN650295
27	*Clostridium* sp. 2NR375.1	96	JQ248567
33	*Clostridium peptidivorans* strain TMC4	100	FJ155851
34	*Clostridium intestinale*	97	AY781385
35	*Clostridium disporicum* strain NML 05A027	100	DQ855943
36;37	*Clostridium tyrobutyricum* strain S1	94; 100	JN241679
38	*Clostridium ljungdahlii* DSM 13528	89	CP001666
48; 49	*Clostridium butyricum* strain T-08B	97; 95	FR734082
53	*Clostridium tyrobutyricum* strain SCTB125	61	JN650290

Table 5. Affiliation of the DGGE bands to bacterial species after sequencing of the 16S rDNA gene

3.3.2. Effect of Fe^{2+} addition

The addition of Fe^{2+} at phase DVII had an influence on the bacterial population in comparison to the seed sludge (phase D6). *L. casei*, *Sporolactobacillus* from the LAB and *C. butyricum* became also dominant, while *Olsenella* was not any more detected. The presence of an extra lactic acid bacterium species though, contradicts the observed reduction of lactic acid production rate. The most probable explanation is that given in 5.3. The clostridial metabolism was shifted from lactic acid production, which is triggered under limitation of Fe^{2+} (Dürre, 2005), to butyrate. Nevertheless, LAB could still influence the system as the production of lactic acid in phase DVII indicates, also for HRT as short as 0.5 d, not like in the case of Kim et al. (2006), who discovered only one lactic acid bacterium (*Bacillus racemilacticus*) and only during the phase with OLR = 60 g COD / (L · d), while for higher or lower OLR no such species was found, although lactate has been produced in all cases, indicating that lactate in the system was a product of *Clostridium* species metabolism, which dominated the system.

In an investigation (Karadag & Puhakka, 2010b) about the influence of Fe^{2+} concentration in the range of 0.5 mg/L to 100 mg/L on a CSTR system fed with glucose and operated at HRT of 0.208 d, OLR of 43.2 g glucose /(L·d) and pH 5 an increase of 71% in hydrogen yield for Fe^{2+} concentration of 50 mg/L was achieved, followed by a fermentation shift from ethanol type to butyric acid type like in this work. In two other works (Wang & Wan, 2008) and (Lee et al., 2001) investigating the influence of Fe^{2+} concentration in batch experiments with vials, optimum concentrations of 350 mg/L and 352.8 mg/L were detected respectively. It seems that there is potential for the optimization of the quantity added to the system.

3.3.3. Symbioses in fermentative hydrogen production systems

There are only a few works that have investigated the bacterial population and the influence of HRT and OLR on hydrogen producing systems by molasses. Ren et al. (2007) studied the influence of pH on the microbial population structure of bio-hydrogen production by molasses in a 2.5 L CSTR seeded with sewage solids and operated at HRT of 0.25 d, OLR between 7 g COD / (L·d) and 30 g COD / (L·d) and at temperature of 35 °C. At pH between 5.5 and 6 mixed ethanol-butyrate fermentation was observed. In this work no ethanol could be detected for HRT of 0.25 d. In the reactor a co-existence of clostridia and LAB was observed like in this work. The bacterial population was dominated by *C. pasteurianum*, *Lactococcus* sp., *Desulfovibrio ferrireducens* together with uncultured species of *Actinobacterium* and *Bacteroidetes*. Chu et al. (2011) on the other hand, did not observe such a co-existence. They affiliated most clones found in a suspended sludge system treating fermented molasses with HRT raging from 0.33 d to 0.083 d to *C. butyricum*, *Megasphaera* sp. and *Corynebacterium glutamicum*. In the case of defined substrate, Kim et al. (2006) obtained the optimum hydrogen yield when a LAB (*Bacillus racemilacticus*) together with *Clostridium* species were dominant in the system.

The dominance of the lactic acid bacteria *Sporolactobacillus*, *Olsenella*, along with hydrogen producing *C. tyrobutyricum, C. butyricum* and *Clostridium sp* strain S6 was observed in a Continuously Stirred Tank Reactor (CSTR) at pH 5.5 and HRT of 1 d yielding 1.24 mol H_2/mol hexose (Wongtanet et al., 2007). The bacterial population examination confirmed in this case a symbiosis between the lactic acid and hydrogen producing bacteria, too. In another work (Ohnishi et al., 2010), in which kitchen waste containing lactate was used as substrate, lactic acid removal was also observed along with carbohydrate degradation during hydrogen production in an Anaerobic Sequencing Batch Reactor. The phylogenetic analysis affiliated the dominant bacteria to the genera of *Lactobacillus*, *Selonomonas*, *Veillonella* and *Megasphaera*, which belong to the phylum of *Firmicutes*, to *Prevotella* genus of the phylum of *Bacteroidetes* and to *Atopobium* and *Bifidobacterium* of the *Actinobacteria* phylum. Hydrogen production has been attributed, due to the absence of *Clostridium* species, to *Megasphaera* by simultaneous utilization of the carbohydrates and the lactate contained in the initial feed and produced by the LAB *Lactobacillus* present in the system and which in return as aerotolerant bacterium consumed residual oxygen for establishing a suitable milieu for the anaerobe *Megasphaera* and supplying it with lactate for hydrogen production.

Affiliation	Phase					
	Seed	Seed	DI	DII	DVI	DVII
	Eubacterium species					
Carnobacterium sp. AT12	**o**					
Lactobacillus fermentum		**o**				
Lactobacillus delbrueckii subsp. *Bulgaricus*		**o**	**o**	**o**		
Uncultured *Burkholderiales* bacterium clone CA38		**o**				
Lactobacillus mucosae					**o**	**o**
Lactobacillus casei						**o**
Sporolactobacillus sp. MB-051				**o**	**o**	**o**
Olsenella sp. oral taxon 809		**o**	**o**			
	Clostridium species					
Clostridium butyricum	**+**	**+**	**+**	**+**	**+**	**+**
Clostridium peptidivorans	**+**					
Clostridium disporicum	**+**					
Clostridium sp. 2NR375.1		**+**	**+**			
Clostridium intestinale			**+**			
Clostridium tyrobutyricum				**+**	**+**	**+**
Clostridium ljungdahlii					**+/-**	**+/-**

Table 6. Allocation of the microbial species to the seed sludge and the operation phases DI to DVIIIe. Microbial population has been simplified to species level. Symbols: "o" no hydrogen production or consumption. "+/-" some strains can produce hydrogen. "+" hydrogen production and "-" hydrogen consumption

In an other investigation (Hung et al., 2011b), it was also suggested that the facultative anaerobes of *Streptococcus* sp. and *Klebsiella* sp. found in the granular sludge of hydrogen producing fermentors seeded with untreated sewage sludge, maintained the strict anaerobic conditions required by the *Clostridium* species for hydrogen production. In the work of Kim et al. (2009), a CSTR seeded with thermally pre-treated sludge at 95 °C for 15 min was operaterd at OLR of 50 g COD / (L·d), HRT of 0.5 d, pH regulated at 5.3 and temperature at 35 °C. In this case also, a symbiosis of hydrogen producing clostridia with the lactic acid bacteria *L. delbruecki* and *Lactococcus lactis* was observed. Such an enhancing symbiosis can not be excluded that also takes place in the system of this work, so that the residual dissolved oxygen of the water added into the system could be removed. More over, the possibility of a secondary fermentation by *C. tyrobutyricum* present in most of the experimental phases and facilitated by the production of lactate by the LAB as described in our previous work (Mariakakis et al., 2011) and later demonstrated (Wu et al., 2012) has to be considered, too.

In any case, this symbiosis reduces the available substrate for hydrogen production. Furhtermore, in this work, it was not beneficial to hydrogen production in the phases with long HRTs, but deteriorated or even completely inhibited it. Lactobacilli possess the potential of inhibiting other microorganisms by different mechanisms. Their fermentation products consist mainly of lactic and acetic acid, which reduce the pH which on its side reduce their dissociation degree. In the presence of oxygen many species such as *L. lactis* and *L. bulgaricus* can produce H_2O_2, which is bacteriocidal for gram negative and bacteriostatic for gram positive bacteria (de Vos, 2009). They can produce bacteriocins, proteinaceous substances with bactericidal effect on microorganisms closely related to the producer. Most probable each *Lactobacillus* species has strains that can produce bacteriocins. Among the organisms that have been found in the present work *L. casei* and *L. fermentum* can produce bacteriocins that are inhibiting other lactobacilli (Dworkin et al., 2006). Nevertheless, it was demonstrated that bacteriocins can hinder non-closely related microorganisms, too. For instance, an inhibition effect of *L. lactis* on *C. tyrobutyricum* due to the excretion of the bacteriocin nisin Z was detected (Rilla, 2003) and of *L. paracasei* on *C. acetobutylicum* and *C. butyricum* (Noike, 2002). It can not be excluded that the lactobacilli present on this work exert a similar effect on the clostridia species. There are also some bacteriocin-like substances produced by lactobacilli, such as bulgarican produced by *L. delbrueckii* subsp. *bulgaricus* that can inhibit a wide range of non related pathogenic gram negative bacteria. *L. delbrueckii*, *L. fermentum* and *L. casei* are also capable of producing bacteriophages that can cause cell lysis (Dworkin et al., 2006). *L. casei*, *L. fermentum* and *L. bulgaricus* have been also identified in this work, so an edverse effect can not be excluded as in several other works has been suggested. It has been proposed that *L. ferintoshensis* and *L. paracasei* present along with *Clostridium* sp. and *Coprothermobacter* sp. in untreated digester sludge negatively influenced hydrogen production (Kawagoshi et al., 2005). The same was suggested for *Sporolactobacillus* sp. that was present in an anaerobic sequencing batch reactor seeded with heat pre-treated sludge and operated at pH 5.0 using sweet sorghum syrup as substrate at OLR of 25 g sugar/(L·d) (Saraphirom & Reungsang, 2011). They justified it by their ability to produce bacteriocins as Noike et al. (2002) suggested. None of these substances has been monitored, so a possible accumulation in concentrations that are inhibiting to the clostridia can only be determined by a general parameter as the COD.

Affiliation	Phase						
	D1	D2	D3	D4	D5	D6	D7
Eubacterium species							
Marine bacterium SJ-BF7	o						
Olsenella sp. oral taxon 809	o		o	o	o	o	
Lactobacillus mucosae		o	o	o	o	o	o
Sporolactobacillus sp. MB-051		o	o	o			
Clostridium tyrobutyricum							+
Clostridium species							
Clostridium ljungdahlii	+/-	+/-	+/-	+/-	+/-	+/-	+/-
Clostridium butyricum		+					
Clostridium tyrobutyricum			+	+	+	+	+

Table 7. Allocation of the microbial species to the seed sludge and the operation phases D1 to D7. Microbial population has been simplified to species level. Symbols: "o" no hydrogen production or consumption. "+/-" some strains can produce hydrogen. "+" hydrogen production and "-" hydrogen consumption

In most cases, heat treatment of the seed sludge was applied, but it was not sufficient to inhibit the growth of lactic acid bacteria, although they can not form spores and it has been demonstrated *in vials* that heat treatment between 50 °C and 90 °C is sufficient (Noike et al., 2002). For instance, *L. delbrueckii* and *L. fermentum* were able to be identified at batch experiments producing hydrogen seeded with activated sludge and digester sludge thermally pre-treated at 65 °C for 30 min (Baghchehsaraee et al., 2010; Hafez et al., 2010). It seems that with increasing inoculum quantity, thermal treatment becomes less effective and it can not hinder the co-dominance and activity of lactic acid bacteria.

4. Conclusion

Hydrogen production by molasses could be successfully carried out in large lab-scale reactors for a period longer than 180 d and under variable combinations of OLR and HRT. The maximum H_2 yield obtained was 1.53 mol H_2/mol hexose for HRt of 1 d and OLR of 36.1 g sucrose/(L·d). Improvement of the hydrogen production yield of 28%was achieved by the addition of Fe^{2+} to an end concentration of 1000 mg/L. In figure 7 the acquired hydrogen yields of all phases as a function of the operation parameters HRT and OLR, along with a suitable range of combination of these parameters, as determined in the current work, are presented. Combinations resulting to COD concentrations higher than 50 g/L (phase D3), was showed to be inhibitory to H_2 production. Reason was not the undissociated form of acids, but most probably the production and accumulation of bacteriocidal or bacteriostatic substances, excreted by the LAB. The second line indicates the combination for which the process becomes unfavorable in terms of substrate utilization efficiency and hence can not be considered as cost effective. The applied seed sludge pre-treatment and reactor start-up methods were successful in enriching the biomass in hydrogen producing microorganisms and killing methanogenic microorganisms that are detrimental to H_2 production. Nevertheless, H_2 production has

been carried out parallel to lactic acid metabolism, which was driven either by the presence of LAB, or by the hydrogen producing clostridia due to iron Fe^{2+} limitation. A co-existence of clostridium species with lactic acid bacteria seems to be unavoidable, even for extensive pre-treatment of the seed sludge. Lactic acid bacteria influence the system primarily by consuming the substrate available for hydrogen production. The co-existence of clostridia and LAB though, seems to become beneficial to hydrogen production at HRTs in the range of 0.5 d to 1 d by supplying certain clostridia genera that are capable of performing secondary fermentation with substrate and/or by removing the residual dissolved oxygen from the system and hence establishing an appropriate milieu for the growth of clostridia. For the successful technical implementation of hydrogen production, for which process control is complex and oxygen in trace concentrations is to be expected, this symbiosis may be regarded as pre-requisite. The exact extent, to which the LAB contribution is beneficial and not adverse, requires the quantification of the biomass for the allocation of the metabolic products to specific microbial species and the monitoring of the concentrations of possible inhibiting substances.

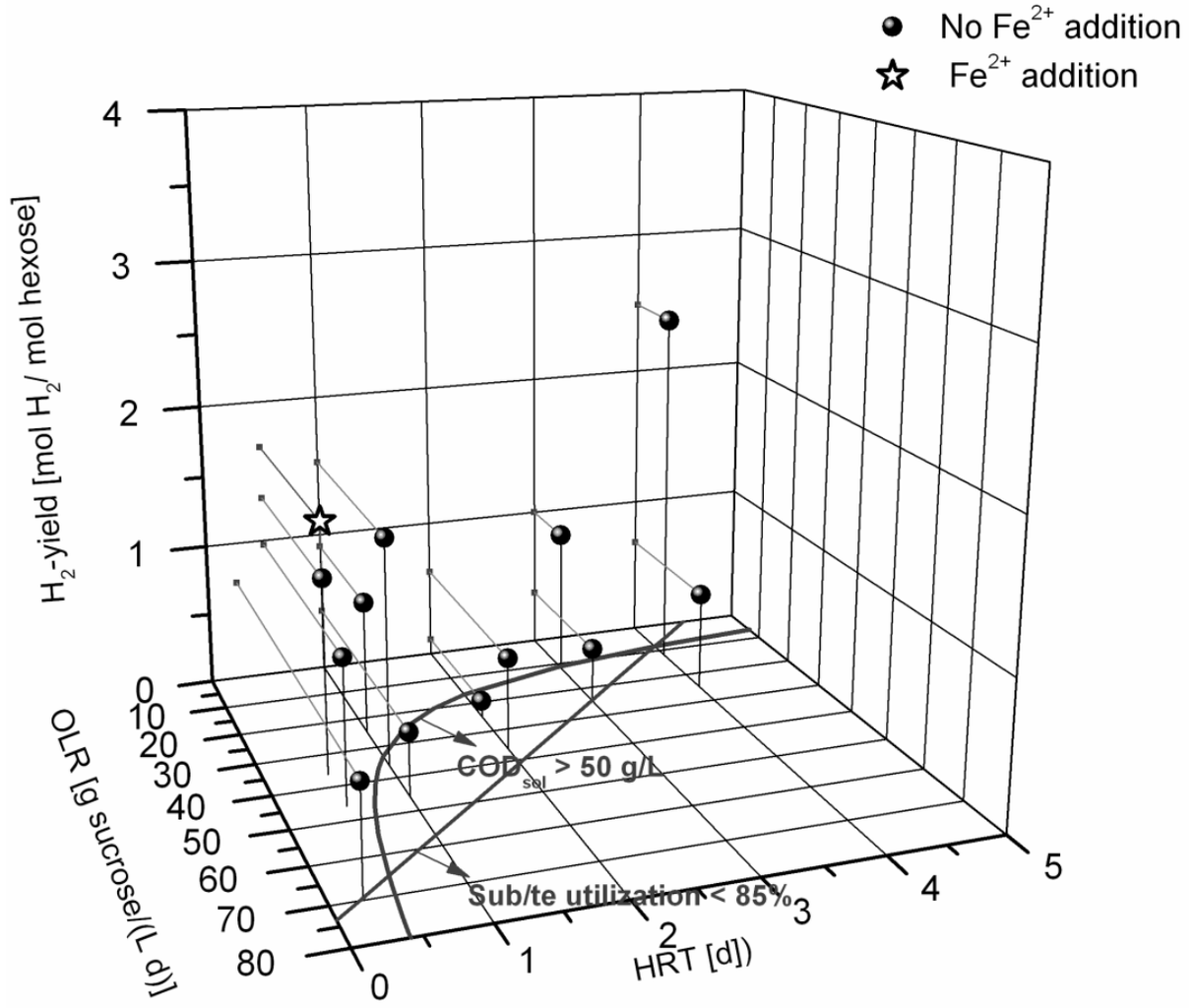

Figure 7. Suitable range of OLR-HRT combination for fermentative hydrogen production by molasses at pH 5.5

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Acknowledgement

This work was supported by the German Federal Ministry of Education and Research (BMBF), EnBW AG, Purolite Deutschland GmbH and RBS wave GmbH, Grant number 03SF0351. We would like to warmly thank Ms. Kerstin Matthies of the Karlsruhe Institute of Technology for the microbial population analyses.

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