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Scale-up and Process development of biological hydrogen process by *Caldicellulosiruptor* species using ‘fibresludge water’

Sudhanshu Pawar

Erika Lönntoft

Karin Willquist

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1. Introduction

The MultiBio project has a goal to establish and demonstrate a novel multipurpose biorefinery cascade concept while producing three renewable biobased products: 1) biohydrogen, 2) biopolymers and 3) protein rich meal ingredients for fish farming. The vision of MultiBio is that the applications of hydrogen produced from pulp and paper industry waste streams has the am to contribute to the environmental goals: Reduced Climate Impact by reducing the use of hydrogen deriving from natural gas that has a CO_2 footprint of production of 12 ton/ton hydrogen gas (H_2) corresponding to about 100 ktons of CO_2 from 320 GWh. The refineries in Sweden contribute to 18% of the CO_2 emissions from industries in Sweden. Reliable supply of biohydrogen gives incentives for a transition to biofuel production in this sector reducing its climate impact many times more than the direct reduction of hydrogen. Clean Air and Natural Acidification Only since FC vehicles have no harmful emissions to the air. Transport in Sweden currently contributes to approximately 40% of its nitrogen oxide and particle emission, which can be reduced by biohydrogen produced from pulp and paper mill residues and other resources. The expected stimulation effect of Multibio on other biohydrogen production routes (due to the upscaling activities) is of great importance in the contribution to this goal.

To achieve these long-term visions the goals MultiBio and milestones of WP 3 are to i) Upscale of the biohydrogen process to pilot scale and production of sufficient effluent for laboratory and industrial feed trails (Milestone 3.1) and ii) Demonstrate a representative biohydrogen process for the production of hydrogen, acetate and protein, tested in a relevant environment that shows evidence of performance on full-scale and where realistic problems have been identified and addressed (TRL 6; Milestone 3.2). This report will discuss the progress towards achieving these Milestones.

Processes at pulp and paper mills generate a variety of effluents which are currently treated via physicochemical and/or biological methods. Interestingly, a fraction of the effluent streams is rich with hemicellulosic sugars. Bacteria belonging to genus *Caldicellulosiruptor* have been shown to utilize and prefer wood-derived sugars to produce H_2. Moreover, earlier work in MultiBio reported successful growth and H_2 production by a microbiome of five different *Caldicellulosiruptor* species in an undiluted but enriched ‘Fibresludge’ process water albeit when grown in small flasks.

On route to commercialization, a technology generally matures through stages that are widely termed as 'Technology readiness levels (TRL)' on a scale from 1 to 9, where the technology is fully commercialized at TRL 9. To the best of our knowledge, *Caldicellulosiruptor* species have never been cultivated in reactors with more than 10 L of active volume. Thus, through MultiBio we attempted to increase the current TRL level of biohydrogen production by *Caldicellulosiruptor* species by - 1) cultivating them in two different types of lab-scale bioreactors using undiluted 'Fibresludge' water, and 2) cultivating them in a reactor with 400 L of active volume albeit using a synthetic medium. The notion of TRL 6 will in biohydrogen production related to cheaper medium and increased hydrogen productivities. Both of which are discussed in this report. Previous report has discussed the challenge of bioprocess producing process in respect to safety regulation. A lot of efforts was therefore put in managing safe hydrogen production (according to the ATEX classification and regulations) during pilot operation. Similar consideration needs to be made for full scale operations.

Moreover, a significant amount of *Caldicellulosiruptor* biomass was needed to be produced to perform fish feed formulation trials at Swedish Agricultural University (SLU), Uppsala. Hence, it was necessary to perform experiment at larger scale to produce as much biomass as possible within the available resources. The experimental methodology used, and the results obtained are disseminated and discussed in this report.

2. Materials and methods

2.1 Microbial strains used and their maintenance

Five different strains of *Caldicellulosiruptor* originally purchased from DSMZ, Germany were provided by the Division of Applied Microbiology, Lund University and were used in the project (Table 1).

Table 1: Strains of Caldicellulosiruptor used in the experiments

Strain DSMZ #	**Taxonomical name**
DSM-8903	*Caldicellulosiruptor saccharolyticus*
DSM-6725	*Caldicellulosiruptor bescii*
DSM-18902	*Caldicellulosiruptor kronotskyensis*
DSM-13100	*Caldicellulosiruptor owensensis*
DSM-18901	*Caldicellulosiruptor hydrothermalis*

The recipe of the growth medium and the protocols for the anaerobic cultivation of strains in crimp-sealed serum flasks were followed as reported previously [1, 2] unless specified otherwise.

2.2 Pilot-operation

2.2.1 Modification of facilities at IndiEnz AB

As reported earlier in the project, due to safety concerns of handling H_2, and as per the 'Directive 1999/92/EC of the European Parliament'[1] a facility with a dedicated ATEX-classified zone was necessary to perform the pilot scale experiments. In addition, all the necessary modifications and rearrangements were done at the facility to comply with the 'Directive 2014/34/EU of the European Parliament'[2], the reactor for instance had to be disconnected from the power supply. A hydrogen gas alarm combined with the Red-Siren (Acandia Mät & Styrteknink AB, Tyresö, Sweden) was installed in the ATEX-classified zone. A liquified N_2 gas tank was installed and connected to facilitate the

[1] https://eur-lex.europa.eu/legal-content/EN/TXT/HTML/?uri=CELEX:31999L0092&from=EN
[2] https://eur-lex.europa.eu/eli/dir/2014/34/oj

stripping of H_2 gas from reactors. By removing the H_2 gas from the reactor, through stripping, the explosion risk is decreased substantially.

2.2.2 Inoculum preparation, medium composition and reactor operation

The inoculum was developed as reported earlier in the project [3] by performing sequential batch cultures of the aforementioned *Caldicellulosiruptor* strains in lab-scale reactors. The cultures were centrifuged, and the pellets were frozen in 30% glycerol solutions at -80° C in crimp-sealed serum flasks. These flasks were used to inoculate the 60 L reactor at Indienz AB. The culture grown in 60 L reactors was used as inoculum for the cultivations in the pilot-scale, 430 L reactor.

The cultivation medium used for inoculum development as well as for pilot-scale cultures contained: Glucose, 10 g; $(NH_4)_2SO_4$, 1.5 g; Urea, 1.5 g; $MgSO_4 \cdot 7H_2O$, 0.4 g; KH_2PO_4, 1 g; yeast extract, 1 g; Cysteine.HCl, 0.4 g, and a modified trace element solution (SL-10) 1 mL [4]. First, the reactor was filled with water and then a concentrated solution of all the chemicals except glucose and cysteine.HCl were added to the reactor. Then the circulation of water from water bath was switched on to heat the reactor to 70 °C. Subsequently, the N_2 sparging was also started at 30-40 L /min for 430 L reactor and at about 5 L / min for 60 L reactor. Once the reactor was heated to 70 °C, concentrated solutions of cysteine.HCl and glucose were added and pH controller was set at pH 6.7 and was switched on. Once the pH and temperature were stable, the inoculum was added to the reactors. In case of the 430 L reactor, a freshly grown inoculum was transferred from the 60 L reactor using a tubing pre-filled with water containing cysteine.HCl and a peristaltic pump. Upon inoculation, a sample was collected to analyse the starting cell density and metabolite concentration.

Cultivations were performed until the growth reached a plateau and then about 90% of culture was harvested into a 1 m^3 tank. The rest of the culture was used as inoculum to restart a fresh cultivation using the medium as described above and following a similar method. In this way, at least 15 sequential batches were performed to harvest a total of about 6 m^3 of culture.

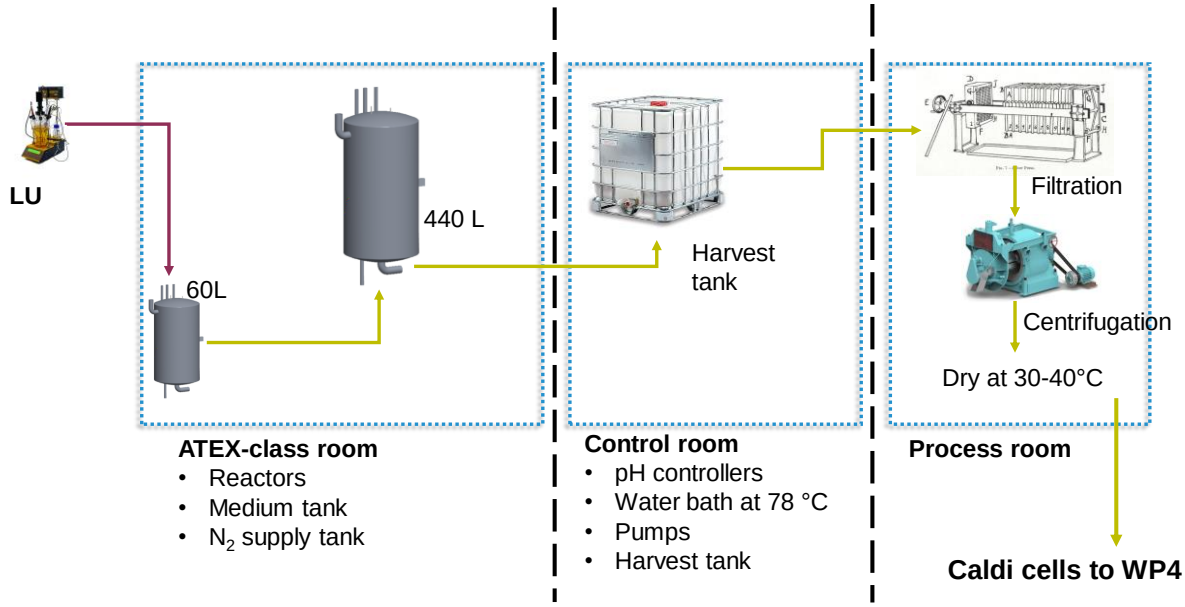

Figure 1 Process set-up at IndiEnz AB. The facilities were distributed in 3 different rooms demarked with dashed lines in black. The pictures of the harvest tank, filtration unit and centrifuge are merely representative.

2.2.3 Filtration, centrifugation and drying of harvested culture

The harvested culture was allowed to cool down to room temperature in the 'harvest tank' (Fig. 1, Appendix 1). Then it was pumped continuously through a crossflow, membrane-filtration unit using membranes with 0.45 µm pore-size. The flow rates were adjusted such that the backpressure did not increase more than 0.7 bar. Upon filtration, the retentate was centrifuged at 5000*g* to obtain a thick pellet of cells. This pellet was then spread over a baking paper and was allowed to dry in the oven at 30° C up to 3 days. the dried biomass was then collected, weighed and packaged for shipping to SLU. Samples were collected regularly from the filtrate as well as from the supernatant after centrifugation to measure the optical density with a spectrophotometer.

2.2.4 Analysis

Samples were collected regularly to follow the growth and pH in the reactor during all the sequential batches. Samples of one of the batch experiments were analysed via HPLC by IndiEnz AB.

2.3 Lab-scale Experiments with Fibresludge water

2.3.1 Filtration of Fibresludge water

As reported earlier, *Caldicellulosiruptor* strains are tolerant to Fibersludge water obtained from Stora Enso mills and successfully fermented the sugars added to the process water [3]. Therefore, further experiments were performed with Fibresludge water. About 20 L of Fibresuldge water was received from Stora Enso mills. The water was filtered using cellulose membranes (Munktell Filtrak™, pore size < 5 µm, particle retention 5-6 µm) connected to vacuum pump. The filtrate and retentate were stored at -20° C until further use.

2.3.2 Reactor set-up and operation

A continuously stirred tank reactor (CSTR) and an Upflow-anaerobic (UA) reactor were used to perform fermentations using filtered Fibresludge water. The CSTR was installed with a stainless-steel cage stuffed with acrylic wool and 197 g retentate of fibresludge water (Fig. 2). The UA reactor was added with 66 g of K1-carriers (Veolia Water Technologies AB, Lund Sweden) and 202 g retentate of Fibresludge water (Fig. 2).

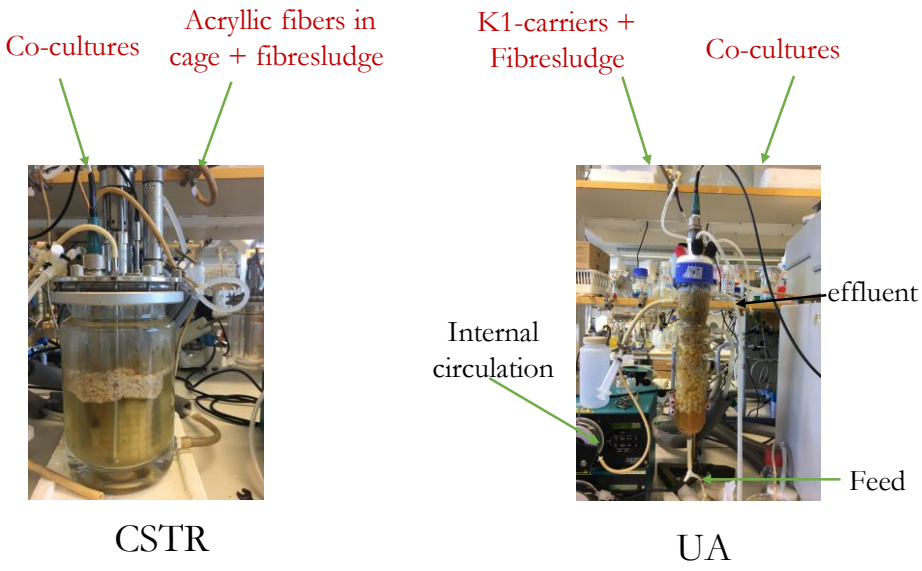

Figure 2 CSTR and UA reactors used in the study

The fermentations were carried out in 7 different phases (Table 1 and 2) with different medium composition and process conditions. For both the reactors, Phases I to IV were operated in semi-continuous mode such that the medium was fed during first 30 hours of the phase and then the reactor was put on the batch mode until the process was switched to the next phase.

To start the cultivations, 590 mL and 1500 mL of synthetic medium containing (g/L)- $(NH_4)_2SO_4$, 1, Urea, 1, $MgSO_4 \cdot 7H_20$, 1, KH_2PO_4, 1, yeast extract, 1, Cysteine.HCl, 0.5, Xylose, 5, and modified mineral solution (1 mL/L) was added to UA and CSTR respectively. Then the mantle of the reactors was connected to a hot water bath maintained at approximately 75 °C, so the temperature in the reactors could be maintained at 70 °C.

Table 2 Experiments performed in CSTR with active volume of 1.5 L.

Phase	Day	CSTR	Mode
I	0 to 2	Filtrate* + Xylose 5 g/L+ macronutrients**+ TE***	Semi-continuous, 1 L
II	2 to 6	Filtrate + arabinose 3 g/L+ macronutrients + TE	Semi-continuous, 1 L
III	6 to 9	Filtrate + macronutrients + TE	Semi-continuous, 1 L
IV	9 to 13	Filtrate + arabinose 3 g/L+ macronutrients + TE	Semi-continuous, 1 L
V	13 to 16	Filtrate + sugar mix¥ + macronutrients + TE	Continuous, HRT = 24 h
VI	16 to 18	dH_2O + sugar mix¥ + macronutrients + TE	Continuous, HRT = 5 h
VII	18	dH_2O + sugar mix¥ + macronutrients + TE	Continuous, HRT = 3.33 h

*Filtrate – filtrate of Fibersludge water; **Macronutrients (g/L)- $(NH_4)_2SO_4$, 1, Urea, 1, $MgSO_4 \cdot 7H_20$, 1, KH_2PO_4, 1, yeast extract, 1, Cysteine.HCl, 1; TE (1 mL/L)– modified mineral solution, ¥ - mixture of Cellobiose 5 g/L, Xylose 5 g/L, and Arabinose 5 g/L.

The CSTR with a working volume of up to 1.5 L were sparged with N_2 at the rate of 66 mL/min to maintain anaerobic conditions as well as to carry H_2 out of the reactor. The pH, stirrer speed and temperature were controlled automatically. The pH was maintained at 6.8 ± 0.5 by addition of NaOH.

The stirrer speed was maintained at 100 rpm. Whereas, for the UA reactor the medium was re-circulated using a peristaltic pump at 100 mL/h. The settings of the automatic pH controller were adjusted so that base was dosed in at very slow rate to allow for a god mixing in the reactor before base is dosed again.

Table 3 Experiments performed in UA with active volume of 590 mL.

Phase	Day	UA	Mode
I	3 to 5	Filtrate* + Xylose 5 g/L+ macronutrients**+ TE***	Semi-continuous, 1 L
II	5 to 9	Filtrate + arabinose 3 g/L+ macronutrients + TE	Semi-continuous, 1 L
III	9 to 14	Filtrate + macronutrients + TE	Semi-continuous, 1 L
IV	14 to 16	Filtrate + arabinose 3 g/L+ macronutrients + TE	Semi-continuous, 1 L
V	16 to 19	Filtrate + sugar mix¥ + macronutrients + TE	Continuous, HRT = 24 h
VI	19 to 20	dH_2O + sugar mix¥ + macronutrients + TE	Continuous, HRT = 5 h
VII	20	dH_2O + sugar mix¥ + macronutrients + TE	Continuous, HRT = 3.33 h

**Filtrate – filtrate of fibersludge water; **Macronutrients (g/L)- $(NH_4)_2SO_4$, 1, Urea, 1, $MgSO_4 \cdot 7H_20$, 1, KH_2PO_4, 1, yeast extract, 1, Cysteine.HCl, 1; TE (1 mL/L)– modified mineral solution, ¥ - mixture of Cellobiose 5 g/L, Xylose 5 g/L, and Arabinose 5 g/L.

2.3.3 Analysis

Headspace samples were analysed for CO_2 and H_2 by gas chromatography, using an Agilent 7890B Series GC (Agilent, Santa Clara, USA) equipped with a TCD detector [5]. The optical density of the culture was measured at 620 nm (OD_{620}) using a Ultraspec-2100 Pro spectrophotometer (Amersham Biosciences, Buckinghamshire, UK). Glucose, acetate, lactate, propionate and ethanol were analyzed by HPLC (Waters, Milford, MA, USA) on an Aminex HPX-87H ion exchange column (Bio-Rad, Hercules, USA) at 45°C, with 5 mM H_2SO_4 (0.6 ml·min^{-1}) as mobile phase. The column was equipped with a refractive index detector (RID-6A; Shimadzu, Kyoto, Japan).

3. Results and Discussion

3.1 Piloting biohydrogen process and production of dried cells

IndiEnz AB have an ATEX-classified zone which could have as large as 440 L capacity reactor and thus was used for the trials (Fig. 1). This allowed for safe operation. The process flow of piloting of biohydrogen process at IndiEnz AB started with medium preparation, by mixing the pre-weighed amounts of required chemicals, dissolving them in sufficient water to get a concentrated solution that was then added to the respective reactor. The cultivation started upon inoculation and interestingly, the *Caldicellulosiruptor* cells grew much faster (μ_{max} = 0.35 h^{-1}, estimated from Fig. 2) than the lab-scale reactor (μ_{max} = 0.19 h^{-1}, estimated from Fig. 3). At least 15 sequential batches were performed in the 430 L-reactor with overall batch time of less than 15 hours for a cell density to reach from about 0.1 to 1.2 – 1.3.

At least 50 L of cell culture was kept in the larger reactor, to be used as an inoculum for the next batch in sequence. Although cultivations were performed in non-sterile conditions, the fast growth rate, short lag phase, and higher temperature may have kept the contaminating microbes from growing in the reactor. Nevertheless, during the sequential batches, freshly grown cells were also added to the larger reactor frequently, to maintain a healthy consortium of *Caldicellulosiruptor* species, as well as to rule out the possibility of enrichment of contaminating microorganisms.

Interestingly, lack of stirring did not affect the mixing and the N_2 bubbling alone was sufficient to maintain the homogeneity in the reactor, as was confirmed by stable pH, temperature and growth in both the reactors (Fig. 2). However, high amounts of NaOH (data not shown) used for maintaining the pH confirmed the observations at lab-scale. It further highlighted the need for finding alternative solution to control pH in the reactor. One of the alternatives could be use of fly ash containing alkali metals [6]. However, its application in biohydrogen process needs to be evaluated in the future.

Caldicellulosiruptor species produced more lactic acid than acetic acid (Fig.2). *C. saccharolyticus* under stress produces more lactic acid than acetic acid [2, 7]. Stoichiometrically, no H_2 is produced when lactic acid is produced. However, when acetic acid is produced, H_2 is produced with maximum efficiency. Thus, it appears that scaling up may have caused stress which resulted in a metabolic shift towards lactate production and thus negatively affected the H_2 production. Additionally, the observed lactic acid could have been produced by *C. owensensis*, as it has a tendency to produce lactic acid even under favorable conditions [8]. During the cultivation, a maximum of 1mole of H_2 was produced per mole of glucose consumed (estimated based on acetate). This is 4 times lower than the maximum theoretical yield.

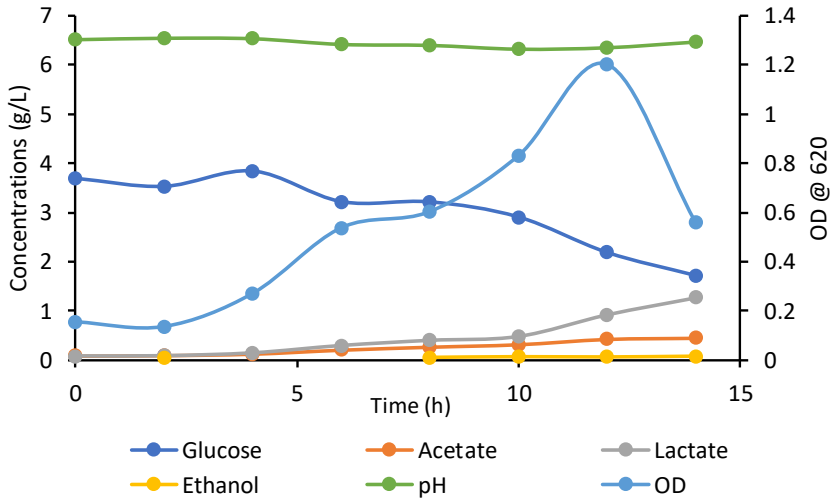

Figure 3 Growth and fermentation profile at pilot scale observed in a batch culture performed in a 430 L reactor.

Analysis of filtrate after membrane filtration and the supernatant that was discarded after centrifugation, together revealed that during the processing of harvested cell-culture, no more than 4% of cells were lost (data not shown). Interestingly, cell loss was noticed due to anomalous behavior of *Caldicellulosiruptor* species during the cultivations at pilot-scale. After reaching a certain cell density (approx. OD_{620} of 1.3), the cell culture suffered a sudden lysis (Fig. 2). This phenomenon was observed during all of the initial sequential batches, and therefore all subsequent cultures were stopped before the cell density increased to more than 1.3. This has never been observed at lab-scale and thus no explanation could be found. However, *Caldicellulosiruptor* species have been suspected to contain elements of prophage i.e. DNA of a bacterial virus [9]. It could be hypothesized that these prophages were activated at large-scale.

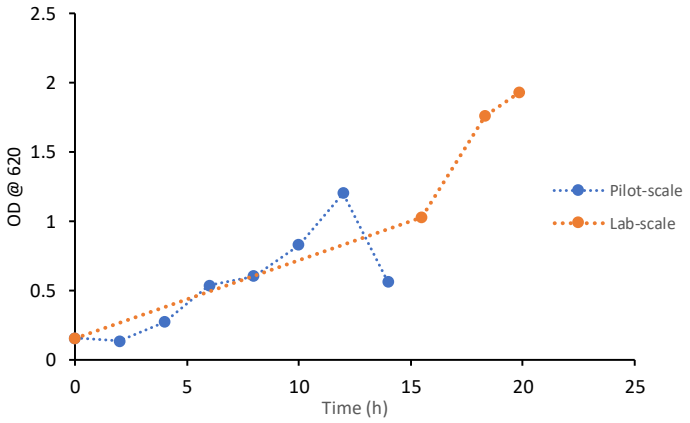

Figure 4 Representative growth profiles of Caldicellulosiruptor species at lab- and pilot-scale.

Overall, the facilities at IndiEnz AB were suitable for the desired biohydrogen process and thus about 6 m^3 of cell culture was obtained and processed, which successfully produced about 2.1 kg of dry cell mass of *Caldicellulosiruptor* species (Fig. 5)

Figure 5 Last batch of dried cells of Caldicellulosiruptor species shipped to SLU

Overall, a biohydrogen process was successfully scaled-up to the pilot scale. Specifically, the method developed for the growth of Caldicellulosiruptor species in large reactors and handling post fermentation is scalable to industrial scale (TRL 8/9). However, as the experiments were performed with the synthetic medium, the potential of H_2 production at large scale was not proven beyond TRL-5. Importantly, challenges such as, low H_2 yield, sudden lysis of cells in batch mode were identified. Identifying such challenges during scale-up could be assigned to a part of process development at TRL-5. The scaled-up biohydrogen process produced feed material albeit for lab-scale experiments of feed trials instead of industrial-scale. Overall, we could conclude that the milestone 3.1 was completed with a greater success than the milestone 3.2.

3.2 Lab-scale biohydrogen production using Fibersludge water

The up-scaling experiment showed an increase in growth rate. However, it indicated a poorer hydrogen productivity and yield. To improve these parameters that are essential for an industrial scale facility with the purpose to generate acetate and hydrogen rather than cells additional lab experiments were performed. Previous report has shown that immobilization increases hydrogen productivity and

allows reactors to be operated at lower retention times i.e. faster process. These results were further explored using effluents from the pulp and paper mills.

The fermentations were performed in UA and CSTR reactors under 7 different phases that differed in the type of medium used and the mode of operation (refer to the Tables 1 and 2 for a detailed description of phases). Reactors were fed with undiluted (but supplemented with nutrients), filtered Fibresludge water from the start-up.

Phases I and II were performed to get the cells acclimatized to the Fibresludge water as well as to grow the *Caldicellulosiruptor* species and promote biofilm formation with the use of carriers, fibres or acrylic wool. Indeed, the both reactors accumulated fermentation products- acetate, ethanol, and lactate (Fig. 4 and 5), which are typically produced by the *Caldicellulosiruptor* species.

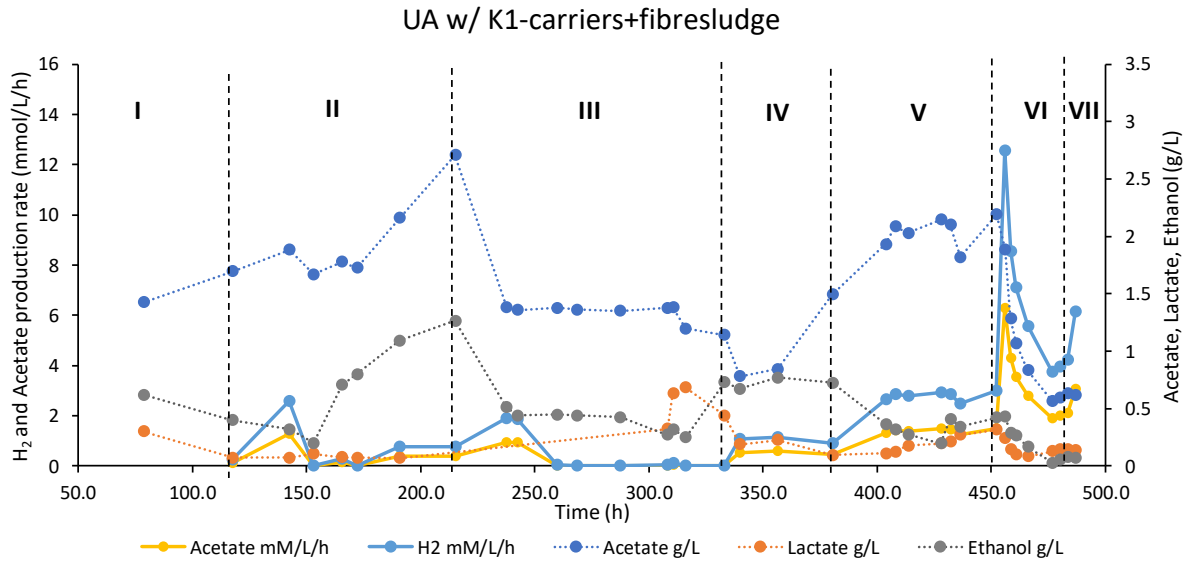

Figure 6 Results of the fermentations performed in UA reactor under seven different phases separated by dashed lines in black (see Appendix 2 for the data). For description of phases please see the Table 2. Phases I to IV were operated in semi-continuous mode such that the medium was fed during first 30 hours of the phase and then the reactor was put on the batch mode until the process was switched to the next phase.

The phases form I to IV were operated in semi-continuous mode such that the Fibersludge medium was fed continuously for about 30 hours at 0.41 mL/min. In other words, during the transition between the phases. For example, between phase II and III, the reactor was on continuous mode. This resulted in a combination of effects - dilution of products accumulated in preceding batch and feeding of fresh medium with sugars probably resulting in their production. To calculate the production rates in UA reactor, an assumption was made that, the concentration of a product after one volume change during this period was accumulated entirely as a result of feeding of new medium. For CSTR, this value was further divided by 2 as only half of its active volume was replaced when 700 mL of new medium was pumped in. Hence, during phase III when no sugars were added to the medium, it appears that UA reactor accumulated no more than 1.3 g/L of acetate (Fig. 4), that corresponded to maximum H_2 production rates of 2 mmol/L/h. Interestingly, during the same phase when reactor was on batch mode, lactate and ethanol levels increases (after 300 h), suggesting further fermentability of Fibresludge water, albeit producing un-intended by-products. Whereas, during phase III in CSTR, after the Fibresludge medium was completely pumped in, the reactor continued to accumulate acetate, further

suggesting the fermentability of Fibresludge water by *Caldicellulosiruptor* species when no sugars were added to it.

Both the reactors were operated in continuous mode during phases V to VII. Unlike the semi-continuous mode, no assumptions were needed to determine the product flows. Overall, CSTR produced more acetate, H_2, lactate and ethanol compared to that of UA. Decrease in HRT from 24 h to 5 h increased productivities. Indeed, it has been seen previously that decrease in HRT increases H_2 productivity [10]

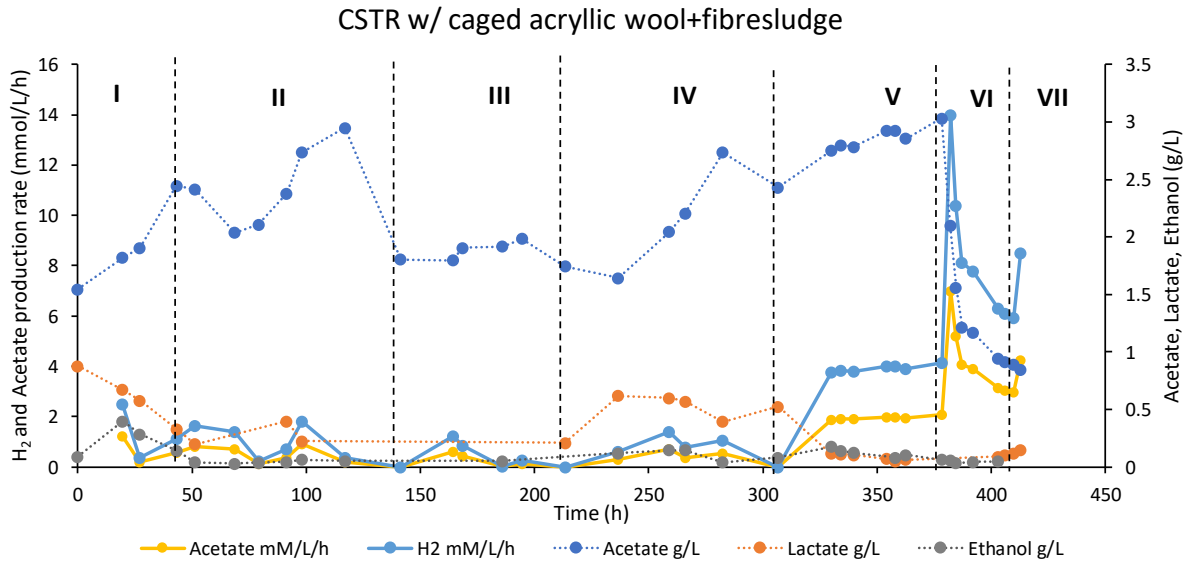

Figure 7 Results of the fermentations performed in 'caged-CSTR' reactor under seven different phases separated by dashed lines in black (see Appendix 3 for the data). For description of phases please see the Table 1. Phases I to IV were operated in semi-continuous mode such that the medium was fed during first 30 hours of the phase and then the reactor was put on the batch mode until the process was switched to the next phase.

The maximum H_2 production rate observed in CSTR was 13.9 mmol/L/h whereas in UA it was 12.6 mmol/L/h when both the reactors were operated at HRT of 5h and when the Fibresludge medium was supplemented with sugars. The addition of sugars – cellobiose, xylose, and arabinose was intended to mimic the concentration of Fibresludge water using a suitable membrane filtration technology. Presumably, membrane filtration would have concentrated the sugars but resulting concentrate would not have inhibited the *Caldicellulosiruptor* species, as they showed no signs of inhibition when grown in undiluted Fibresludge water. With this assumption, and the maximum H_2 productivity obtained during the phase V, it was estimated that a stable biohydrogen process performed in UA reactor with 10 m^3 working volume, using concentrated Fibresludge water could produce H_2, sufficient for 1 FCEV car driven from RISE office in Lund to Gothenburg's Central station (see Appendix 4 for calculations). Arguably, at least 10 times higher H_2 production rate would be required to make the biohydrogen process industrially relevant.

Similar to pilot-scale reactor, Nitrogen stripping alone was able to maintain the homogeneity and pH in the reactor. No biofilm was observed on the carriers in the UA reactor or on the acrylic wool. Biofilm formation on carriers or supporting materials such as acrylic wool, needs reactors to be operated for significantly longer time than 20 days. Indeed, when Caldicellulosiruptor cells were reported to form biofilm previously, the reactor was operated for 2 months [10]. Moreover, no

significant degradation of Fibresludge (that was added as carrier material for the bacteria) was observed in either of the reactors. Arguably, a high cell-density culture treating a stream with high sugar concentration, for a significantly longer time would first establish a healthy biofilm on the fibres and then would degrade it simultaneously. Certainly, lack of biofilm formation, contributed to lack of degradability of fibres, despite previous reports of *C. saccharolyticus* species degrading plant derived biomass [11].

4. Conclusions

Scale-up from lab-scale to pilot-scale did not pose any challenges for growing the *Caldicellulosiruptor* cells. The method used could be conveniently scaled-up to even larger, industrially relevant scale. A little more than 2 kg of dry cells of *Caldicellulosiruptor* species were delivered to the SLU to perform further experiments with fish feed formulations.

Caldicellulosiruptor species were able to ferment the sugars naturally present in Fibresludge water but were not able to demonstrate the degradability of fibres in Fibresludge. A process treating concentrated Fibresludge water could produce H_2 at a significant rate, which could further be evaluated via technoeconomic analysis for its relevance.

The ability for the cells to produce hydrogen at low HRT of 3.3h shows promise in using the system in high rate water system without taking up to large surface. It can be compared to biogas plant that can have residence times between 12-24h in fast operation systems.

Overall, through this work, the biohydrogen process using *Caldicellulosiruptor* species was scaled-up to TRL-5 producing feed for further experiments.

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Appendix 1

Pilot-scale reactors at Anneberg. The larger one is of the 430 L working volume.

Harvest tank

Appendix 2

Data from UA reactor.

2019-10-10

sample nam	h	glucose	acetate	lactate	ethanol	ac mmol/l/h	H2 mM/L/h	
m9_7	78.4	0.068	1.427	0.299	0.617			semi-continuous, fibresludge medium 100%, xylose 5 g/L
m9_10	117.5	0.108	1.697	0.07	0.397	0.12	0.23	
m9_11	142.5		1.882	0.072	0.317	1.29	2.57	semi-continuous
m9_12	152.9		1.67	0.105	0.198	0.00	0.00	0,41 mL/min for 20
m9_13	165.4		1.779	0.076	0.705	0.15	0.29	hours, fibresludge
m9_14	172.0		1.724	0.072	0.799	0.00	0	medium 100%,
m9_15	190.8		2.16	0.067	1.09	0.39	0.77	arabinose 3 g/L
m9 16	215.2		2.71		1.263	0.38	0.75	
m9 17	237.6		1.385		0.509	0.95	1.89	semi-continuous,
m9 18	242.4		1.358		0.438	0.93	1.86	fibresludge medium
m9 19	259.9		1.375		0.446	0.02	0.03	100%, no added
m9_21	268.5		1.362		0.44	0.00	0.00	sugar
m9_22	287.3		1.35		0.422	0.00	0.00	
m9_23	308.2		1.375	0.323	0.27	0.02	0.04	
m9_24	310.5	0.093	1.382	0.633	0.318	0.05	0.10	
m9_25	316.0	0.067	1.195	0.684	0.246	0.00	0	
m9_26	333.0	0.067	1.14	0.438	0.729	0.00	0	
m9_27	339.8	0.065	0.778	0.186	0.671	0.53	1.06	semi-continuous,
m9_28	356.4	0.099	0.843	0.224	0.769	0.58	1.15	fibresludge medium
m9_29	380.4	0.094	1.493	0.095	0.723	0.45	0.90	100%, arabinose 3 g/L
m9_30	403.8	0.075	1.933	0.11	0.365	1.32	2.64	continuous mode
m9_31	408.0	0.078	2.084	0.122	0.317	1.42	2.85	HRT- 24 h, 100%
m9_32	413.6	0.075	2.028	0.176	0.275	1.39	2.77	fibresludge
m9_32a	428.0	0.079	2.145	0.193	0.196	1.47	2.93	medium, cellobiose,
m9_33	432.0	0.078	2.1	0.208	0.408	1.44	2.87	xylose, Arabinose
m9_34	436.4	0.07	1.82	0.271	0.342	1.24	2.49	
m9_35	452.3	0.074	2.191	0.316	0.421	1.50	2.99	
m9_36	456.0	0.075	1.886	0.24	0.426	6.29	12.57	continuous mode
m9_37	458.4		1.284	0.143	0.29	4.28	8.56	HRT - 5h, synthetic
m9_38	461.0		1.065	0.096	0.265	3.55	7.10	medium, cellobiose,
m9_39	466.0		0.834	0.083	0.163	2.78	5.56	xylose, Arabinose
m9_40	476.8		0.563	0.128	0.026	1.88	3.75	
m9_41	480.1		0.595	0.141	0.055	1.98	3.97	
m9_42	483.6		0.632	0.147	0.077	2.11	4.21	
m9_43	486.8		0.614	0.139	0.072	3.07	6.14	cont. Mode - hrt -3,33

Appendix 3

2019-10-10

Data from CSTR

sample nam	h	glucose	Acetate g/	Lactate g/l	Ethanol g/	Acetate mM	H_2 mM/L/h	
m10_1	0	1.201	1.546	0.878	0.09			semi-continuous,
m10_2	19.58333		1.819	0.672	0.399	1.24	2.49	fibresludge medium
m10_3	27.25		1.907	0.579	0.287	0.19	0.38	100%, xylose 5 g/L
m10_4	43.33333		2.447	0.332	0.143	0.56	1.12	
m10_5	51.33333		2.413	0.198	0.042	0.82	1.65	semi-continuous,
m10_6	68.75		2.038		0.029	0.70	1.39	fibresludge medium
m10_7	79.08333		2.108		0.037	0.11	0.23	100%, xylose 5 g/L
m10_8	91.55		2.378	0.397	0.045	0.36	0.72	
m10_9	98.16667		2.736	0.227	0.064	0.90	1.80	
m10_10	117	0.06	2.947		0.054	0.19	0.37	
m10_11	141.3333	0.064	1.805			0.00	0.00	
m10_12	164.25	0.064	1.797			0.61	1.23	semi-continuous,
m10_13	168.4667	0.061	1.905			0.43	0.85	fibresludge medium
m10_14	186.05		1.917		0.054	0.01	0.02	100%, no added
m10_15	194.5667	0.059	1.983			0.13	0.26	sugar
m10_16	213.4167		1.743	0.211		0.00	0.00	
m10_18	236.6667		1.643	0.62	0.122	0.30	0.60	semi-continuous,
m10_19	259.0833		2.046	0.6	0.147	0.70	1.40	100% fibresludge
m10_20	265.9167		2.204	0.57	0.146	0.39	0.77	medium, cellobiose,
m10_21	282.5	0.076	2.734	0.393	0.043	0.53	1.07	xylose, Arabinose
m10_22	306.6667		2.431	0.524	0.081	0.00	0.00	
m10_23	330		2.75	0.118	0.178	1.88	3.76	continuous mode
m10_24	334.1667		2.799	0.113	0.142	1.91	3.83	HRT- 24 h, 100%
m10_25	339.75		2.782	0.104	0.125	1.90	3.80	fibresludge
m10_26	354.1667		2.923	0.07		2.00	3.99	medium, cellobiose,
m10_27	358.1667		2.922	0.053	0.084	2.00	3.99	xylose, Arabinose
m10_28	362.5833		2.855	0.063	0.105	1.95	3.90	
m10_29	378.4167		3.029		0.065	2.07	4.14	
m10_30	382.1667		2.096		0.061	6.99	13.97	continuous mode
m10_31	384.5833		1.559		0.033	5.20	10.39	HRT - 5h, synthetic
m10_32	387.1667		1.217			4.06	8.11	medium, cellobiose,
m10_33	392.1667		1.167		0.047	3.89	7.78	xylose, Arabinose
m10_34	402.8333		0.946	0.092	0.05	3.15	6.31	
m10_35	406.1667		0.916	0.106		3.05	6.11	
m10_36	409.75		0.888	0.121		2.96	5.92	
m10_37	412.9167		0.848	0.152		4.24	8.48	cont. Mode - hrt -3,33

Appendix 4

gasoline 32.2 MJ/L

2019-10-10

Lund-Göteborg	280	km
	22.4	L gasoline
	721.28	MJ energy needed

max. H2 production rate in UA

12.57	mol/m3/h	
0.0251467	kg/m3/h	
306.79	ml/L/h	=L/m3/h
0.31	m3/m3/h	
0.03	kg/m3/h	
3.31	MJ/m3/h	
79.52	MJ/m3/day	

		LHV	
H2	2g/mol	120	MJ/kg

How many cars can drive to Gothenburg with H2 produced from 1 m3 reactor in a day	**0.11**

Assumption: Energy efficiency of ICE car is the same as FCEV.