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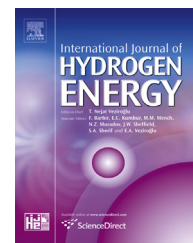
Biohydrogen production from wheat straw hydrolysate using *Caldicellulosiruptor saccharolyticus* followed by biogas production in a two-step uncoupled process[☆]

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ABSTRACT

A two-step, un-coupled process producing hydrogen (H_2) from wheat straw using *Caldicellulosiruptor saccharolyticus* in a 'Continuously stirred tank reactor' (CSTR) followed by anaerobic digestion of its effluent to produce methane (CH_4) was investigated. *C. saccharolyticus* was able to convert wheat straw hydrolysate to hydrogen at maximum production rate of approximately 5.2 L H_2/L/Day. The organic compounds in the effluent collected from the CSTR were successfully converted to CH_4 through anaerobic digestion performed in an 'Up-flow anaerobic sludge bioreactor' (UASB) reactor at a maximum production rate of 2.6 L CH_4/L/day. The maximum energy output of the process (10.9 kJ/g of straw) was about 57% of the total energy, and 67% of the energy contributed by the sugar fraction, contained in the wheat straw. Sparging the hydrogenogenic CSTR with the flue gas of the UASB reactor ((60% v/v) CH_4 and (40% v/v) CO_2) decreased the H_2 production rate by 44%, which was due to the significant presence of CO_2. The presence of CH_4 alone, like N_2, was indifferent to growth and H_2 production by *C. saccharolyticus*. Hence, sparging with upgraded CH_4 would guarantee successful hydrogen production from lignocellulosic biomass prior to anaerobic digestion and thus, reasonably high conversion efficiency can be achieved.

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1. Introduction

Foreseeing the advancements in the energy infrastructure and end-user technologies, the growth in world energy consumption can be expected to slow down [1]. However, the supply of fossil fuels is expected to hit rock-bottom in coming decades [1]. Moreover, un-restrained usage of fossil fuels has contributed to growing concern over global warming. Hence, it

is more than evident that the world needs alternative, renewable energy sources which should also be environmental friendly.

Of late, agricultural residues are increasingly being considered as a potential source of renewable biomass. Estimations of agricultural residues are about 10^{10} tons/year globally, corresponding to 4.7×10^{10} GJ of energy (about 9% of the global energy consumption in 2008 [1]), and about two-thirds consists of cereal residues [2]. Wheat straw is a lignocellulosic biomass, consisting of 35–40% cellulose, 20–30% hemicelluloses and 8–15% lignin [3]. These sugars can potentially be used in microbial fermentations to produce biofuels, such as, bioethanol, biogas and hydrogen. So far, however, bioethanol production from lignocellulosic biomass has not been successful enough due to a variety of techno-economic challenges [4–6]. Alternatively, studies have shown efficient production of hydrogen (H_2) from wheat straw hydrolysate (WSH) by dark fermentation (DF) [7–9]. H_2 is widely considered as a fuel of the future due to its properties of rapid burning speed, no emissions of greenhouse gases, higher energy density, low minimum ignition energy and a very high research octane number [10–13]. Currently, H_2 is mainly produced by reforming fossil fuels making it a non-renewable and non-carbon neutral, which is in contrast to what DF of agricultural residues has to offer. The thermophilic *Caldicellulosiruptor saccharolyticus* possesses the ability of producing H_2 via DF at yields near the theoretical maximum of 4 mol H_2/mol of hexose consumed [14]. In addition, *C. saccharolyticus* can naturally ferment a wide range of poly-, oligo- and mono-saccharides including sugars present in lignocellulosic hydrolysate [15]. Moreover, the absence of 'carbon catabolite repression' enables it to co-ferment glucose, xylose and arabinose among other sugars [16].

During the DF, the highest theoretical maximum yield of H_2 can be obtained only when acetate is the major by-product [17]. The latter, contains as much as 67% of the total energy present in the substrate. This energy can be retrieved in the form of H_2 by either photo-biological process or microbial electrolysis, which are both, however, still under development [18]. Alternatively, the effluent from DF can be transferred to an anaerobic digester, wherein acetate can be converted to CH_4 by acetoclastic methanogenesis, which is a reliable and an industrially established process [3,18]. Various studies of combined H_2 and CH_4 production in a two-step process have been reported in recent years [9,19]. Furthermore, H_2 and CH_4 together can give a mixture termed *hythane*, which has superior combustion properties compared to CH_4 alone [20].

So far, DF has been carried out largely in a continuously stirred tank reactor (CSTR), in which sparging is needed to actively remove hydrogen to keep the hydrogen partial pressure (p_{H_2}) to a minimum [21,22]. Nitrogen is usually used for sparging at lab-scale, as it is a cheap and inert gas. However, separation of N_2 from H_2 is tedious and thus not exploitable at industrial scale. As an alternative, CO_2 is relatively easier to separate from H_2, but has a detrimental effect on growth of *C. saccharolyticus* [23]. Finally, the CH_4 produced in the anaerobic digestion (AD) can, in principle, be used as sparging gas in the DF, producing *hythane*, after removal of CO_2.

The ability of *C. saccharolyticus* to ferment wheat straw was observed previously [7]. However, since the experiments were

performed on raw wheat straw, they were continued for long duration (about 45 days [7]), which makes it economically unfeasible. On the other hand, various pretreatment methods can generate by-products which may inhibit microbial growth [24,25]. Hence, in this study, we demonstrate the fermentability of pre-treated wheat straw by *C. saccharolyticus* and its ability to sustain growth in the presence of CH_4. We also demonstrate the feasibility of the two-step process, wherein, the wheat straw hydrolysate (WSH) is fermented to produce H_2 in a CSTR by *C. saccharolyticus* and the effluent produced is converted to CH_4 by methanogens in a UASB reactor. During this study, the reactors performing DF and AD were uncoupled. Ideally, however, both the reactors should be coupled together as described previously [26].

2.   Materials and methods

2.1.   Wheat straw hydrolysate

WSH was produced by steam acid pretreatment and enzymatic hydrolysis of wheat straw obtaining an energy content of 11.9 MJ/kg of dry matter (DM) in the WSH. Glucose and xylose were the main sugars and the chemical oxygen demand (COD) was estimated to be 196 g/l. The detailed composition of the hydrolysate has been reported previously [27]. The pre-treated hydrolysate was centrifuged for 15 min at 4900 rpm to remove any remaining solid matter. Subsequently, the supernatant is then allowed to pass through a Whatman's no.1 filter paper supported by a nylon membrane to get rid of insoluble particulate matter. The pH of this clarified hydrolysate was adjusted to pH 7 with 12.5 M NaOH. The filtered neutral hydrolysate was sterilized by filtration using disposable Acrocap™ (pore size – 0.2 μm) filters and the filtrate was collected in sterile screw cap bottles and stored at −20 °C until further use.

2.2.   Microorganism and culture medium

C. saccharolyticus DSM 8903 was purchased from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (Braunschweig, Germany). A modified DSM 640 medium was used as a base medium for all cultivations throughout this work [23]. Routine subcultures and inoculum development were conducted in 250 mL serum bottles containing 50 mL of medium under a N_2 atmosphere. Anoxic solutions of glucose, xylose and arabinose were autoclaved separately and were added to the sterile medium at the required concentration. Filter sterilized WSH was added to a sterile serum bottle and was kept under a N_2 atmosphere.

2.3.   Experimental set-up and operation

Batch cultures of dark fermentation were carried out at 70 °C using 250-mL serum flasks containing 50 mL liquid medium. The preparation of anaerobic flasks was as follows: the modified DSM 640 medium without the carbon source was added to the flasks and thereafter, the flasks were sealed with butyl stoppers and aluminium crimps. Subsequently, the headspace of the flasks was flushed with N_2 unless stated

otherwise. Two separate batch tests were performed: *a*) fermentability test of WSH and *b*) effect of CH_4 present in the headspace on the growth of *C. saccharolyticus*. In the former, four different concentrations of hydrolysate (v/v), 20%, 10%, 6.66% and 5%, were studied. Flasks containing 6.66% and 5% hydrolysate were complimented with pure sugars (glucose, xylose and arabinose) to keep the total sugar concentrations at the level present in 10% v/v WSH (i.e. in g/L glucose, 6.7, xylose, 3.7, and arabinose, 0.4). In test '*b*', the headspace of the flasks was flushed with either CH_4 or N_2. 10% v/v of hydrolysate was used as substrate and a medium with pure sugars was used as control. During all batch experiments, samples were collected at regular time intervals for the determination of biomass, H_2 accumulation and metabolite concentrations. Experiments were continued until H_2 accumulation ceased in the headspace.

The chemostat cultures were carried out as described previously [22] except for the following modifications. In continuous mode, the reactor was fed with a fresh medium containing (per litre of deionised water) NH_4Cl 0.9 g, $MgCl_2.7H_2O$ 0.4 g, KH_2PO_4 0.75 g, K_2HPO_4 1.5 g, Yeast extract 1 g, resazurin 1 mg, trace element solution SL-10 [28] 1 mL and WSH (10% v/v) as a substrate but omitting cysteine-HCl. WSH at 10% v/v contained approximately 11 g/L of total monosaccharide sugars with 23 mg/L of 5-(hydroxymethyl)furfural (HMF) and 114 mg/L of furfural [27]. The reactor was sparged with either 100% N_2 or a gas mixture containing $N_2 + CO_2$ (60%:40% v/v) at the flow rate of 6 L/h. The steady states were obtained at four different conditions, i.e. *Case I*, low growth rate ($D = 0.05$ h^{-1}), N_2 sparging; *Case II*, higher growth rate ($D = 0.15$ h^{-1}), N_2 sparging; *Case III*, low growth rate ($D = 0.05$ h^{-1}), sparging with a mixture of N_2 (60% v/v) and CO_2 (40% v/v); and *Case IV*, higher growth rate ($D = 0.15$ h^{-1}), sparging with a mixture of N_2 (60% v/v) and CO_2 (40% v/v). The steady states were determined after at least five volume changes based on the stability of CO_2 and H_2 levels and biomass concentration. The effluent generated from the chemostat was collected, mixed together and stored at 4 °C before use in AD.

Batch cultures of AD were performed in triplicates using the effluent from DF. The flasks were incubated at 37 °C for 31 days. The experimental procedure and set-up was as described earlier [27,29]. Methane production using the effluent of dark fermentation was performed in UASB reactors in duplicate and under mesophilic (37 °C) conditions. The active reactor volume was 0.8 L and the up-flow velocity was 0.08 and 0.09 mL/h. The rest of the reactor configuration was as previously described [30]. A modified basic anaerobic nutrient solution (BA) was used to supplement the effluent [31], in that, ammonium chloride was substituted with Urea (1 g/L), as the latter is a rich nitrogen source and also a buffering agent. The effluent collected from DF had a pH of 6.6 and a COD of 16.2 g/l before addition of the BA medium. After addition of the BA medium, the pH and the COD changed to 6.9 and 15.3 g/l, respectively (Table 2). Prior to the treatment of the DF effluent, the UASB reactor was continuously fed with the WSH containing about 10 g/l of fermentable sugars. When the feed was switched to DF effluent, the reactors were operated at an OLR of 5.0 g COD/L/day (HRT of 2 days) until they reached stability. Increase in the organic loading rate was performed

by decreasing the hydraulic retention time (HRT). The HRT was decreased from 2.5 to 1.5 days and corresponded to an increase in OLR of 6.0–10.5 g COD/L/day. The treatment period was 49 days.

2.4. Analytical methods

For dark fermentation, gas in the headspace of the serum flasks and the CSTR was analysed for CO_2 and H_2 by gas chromatography, using a dual channel Micro-GC (CP-4900; Varian gas chromatography, Middelburg, The Netherlands), as previously described [28]. The results were analysed with a Galaxie Chromatography workstation (v 1.9.3.2). The optical density of the culture was measured at 620 nm using a U-1000 spectrophotometer (Hitachi, Tokyo, Japan). The cell-free culture medium was used as a blank while measuring the optical density of the cultures. The cell dry weight was determined as previously described [32]. The metabolites, sugars, 5-(hydroxymethyl)furfural and furfural in DF were analysed by HPLC (Waters, Milford, MA, USA) as described previously [22].

The samples collected during anaerobic digestion were analyzed for pH, COD, NH_4^+–N, partial and total alkalinity, volatile fatty acids, gas volume and composition. Methods of sample collection and analysis for the methane potential batch test and UASB reactor were as previously described [27]. The volume of methane and hydrogen were corrected for using the standard conditions (0 °C, 1 atm).

2.5. Calculations

The volumetric H_2 productivity (mM/h) was calculated using the ideal gas law and the H_2 and CO_2 concentrations in the headspace of the serum flasks or CSTR. In case of the CSTR, the calculations were based on the flow rate of the effluent gas and the accompanying partial pressures of H_2 and CO_2. In case of serum flasks, the product gas was allowed to accumulate in the headspace, which is the basis for the calculation. The energy output for each of the cases was calculated based on lower calorific values (LCV) and the quantity of H_2 or CH_4 produced. The LCV for H_2 and CH_4 are 122 and 50.1 MJ/kg, respectively [33].

3. Results

3.1. Fermentability of wheat straw hydrolysate in DF

Media containing 10% or lower levels of WSH showed comparable biomass and H_2 yields, (Fig. 1(A)). Even though, the differences observed were insignificant, yet a decreasing trend can be observed in maximum obtainable H_2 productivities with increasing WSH concentration (Fig. 1(A)). Hardly any or no significant growth and H_2 accumulation was observed in the flasks containing 20% WSH (data not shown). Interestingly, H_2 accumulation and cell growth appears to be enhanced in WSH compared to a medium with only pure sugars (Figs. 1(A) and 2). For obvious reasons, 10% v/v of WSH was added in a growth medium used in further experiments.
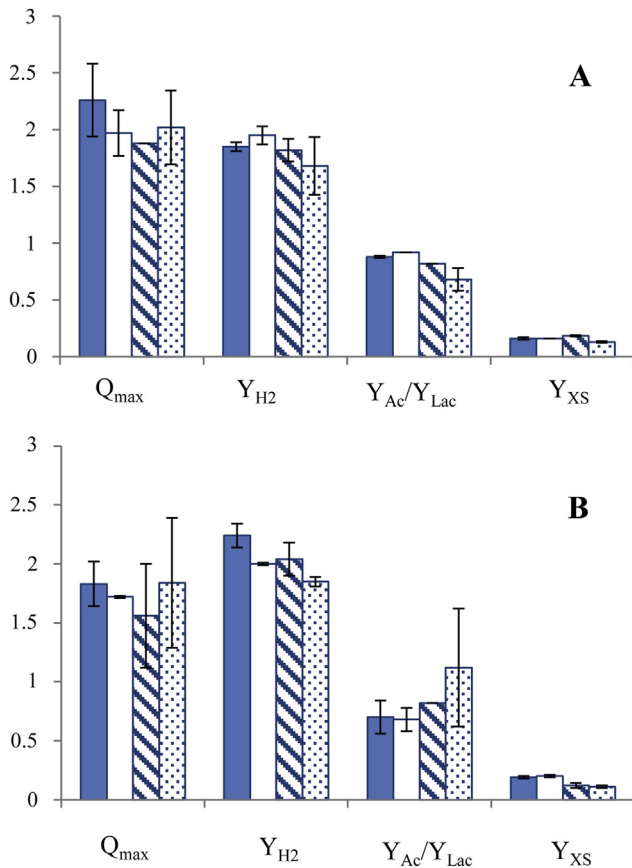
Fig. 1 – Results of the batch fermentations in DF to evaluate the fermentability of WSH by C. saccharolyticus. Q_{max} – maximum H_2 productivity (mmol/L/h); Y_{H_2} – hydrogen yield (mol/mol glucose consumed). Y_{Ac}/Y_{Lac} – the ratio of acetate yield to lactate yield; Y_{xS} – the biomass yield (mol/mol glucose consumed). (A) Fermentability of WSH (v/v), 5% (▨), 6.67% (☐), 10% (▨) and pure sugars (▨). (B) Fermentability of 10% (v/v) WSH in presence of either CH_4 or N_2 in the headspace, 10% WSH + CH_4 (▨), 10% WSH + N_2 (☐), Pure sugars (glucose, xylose and arabinose) + CH_4 (▨) and Pure sugars + N_2 (▨).

3.2. Growth of C. saccharolyticus in presence of methane

H_2 productivities and biomass yield seemed to be unaffected by CH_4 (Figs. 1(B) and 2). Interestingly, the flasks containing 100% CH_4 in the headspace appeared to have slightly higher H_2 yields compared to those containing 100% N_2 in the headspace (Figs. 1(B) and 2). Yet again, the flasks containing WSH showed relatively better biomass formation and H_2 accumulation at a higher maximum growth rate than those containing only pure sugars (Figs. 1(B) and 2). All batch experiments displayed co-consumption of glucose, xylose and arabinose. However, xylose was the most preferred substrate regardless of the growth conditions (Fig. 2).

Although, CH_4 is slightly beneficial; for safety reasons, N_2 was used in all following experiments instead, as both do not affect the performance of C. saccharolyticus negatively. Thus,

the gas mixture of $N_2 + CO_2$ was assumed to mimic the non-upgraded flue gas ($CH_4 + CO_2$) from the AD (Case III and IV). Similarly, cultures sparged with N_2 were assumed to be the same as if sparged with CH_4 (Case I and II).

3.3. Growth of C. saccharolyticus on WSH in controlled bioreactors

In chemostats, four different experimental conditions were employed (using the growth rate and sparging gas composition as variables, Cases I to IV), with a medium containing 10% WSH as carbon source. Out of the four conditions studied, a low growth rate ($D = 0.05 \text{ h}^{-1}$) and sparging the reactor with N_2 resulted in the highest H_2 yield and best of substrate conversions (Table 1). The substrate conversion efficiency decreased with increasing growth rate and when CO_2 was present in the sparging gas. Surprisingly, at a higher growth rate ($D = 0.15 \text{ h}^{-1}$), the culture sparged with $N_2 + CO_2$ displayed a higher H_2 yield and higher specific H_2 production rate than the one sparged with N_2 (Table 1). Also, the highest lactate yield per mole of hexose was observed in the latter case compared to the other conditions. However, the average volumetric H_2 productivity was about 40% higher in the reactors sparged with N_2 only (Table 1, 5.1 L H_2/L/day) than the reactors sparged with $N_2 + CO_2$ (Table 1, 2.9 L H_2/L/day). The overall conversion of substrate in the dark fermentation was found to be in the range of 19–88% (Table 1). Regardless of the growth conditions the culture was able to reduce the potential growth inhibitors (5-(hydroxymethyl)furfural and furfural) present in the WSH (Table 1). Cultures sparged with $N_2 + CO_2$ displayed higher medium osmolalities than their counterparts performed with N_2 sparging (Table 1). Similarly, low amounts of biomass were obtained in chemostats sparged with $N_2 + CO_2$ which were accompanied by higher amounts of residual sugars and consequently lower conversions. The specific consumption rate for xylose was significantly higher than that for glucose in the cultures sparged with $N_2 + CO_2$ (Case III and IV, Table 1), whereas the opposite was true for the cultures sparged with N_2 (Case I and II, Table 1). Carbon and redox recovery was significantly higher than 100% in all the cases studied (Table 1).

3.4. Production of methane from the effluent collected from DF

During anaerobic digestion of the collected DF effluent, an increase in the organic loading rate from 6.0 to 10.5 g COD/L/day resulted in an increase in methane productivity (Table 2). Further increase in the organic loading rate to 15.4 g COD/L/day (1.0 day HRT) resulted in an increased methane production rate, i.e. 3.95 L/L/day, after 6 days of treatment time (data not shown). At a stable organic loading rate of 10.5 g COD/L/day (equivalent to 1.5 days HRT) a maximum methane production rate of 2.64 L/L/day (Table 2) was observed. The methane yield ranged from 0.28 to 0.26 L/g COD independent of the OLR and the methane content in biogas was about 60% (Table 2).

Stable operational conditions prevailed throughout the entire treatment period. The pH remained stable at around 7.50 for all applied OLRs. The effluent of the UASB reactor
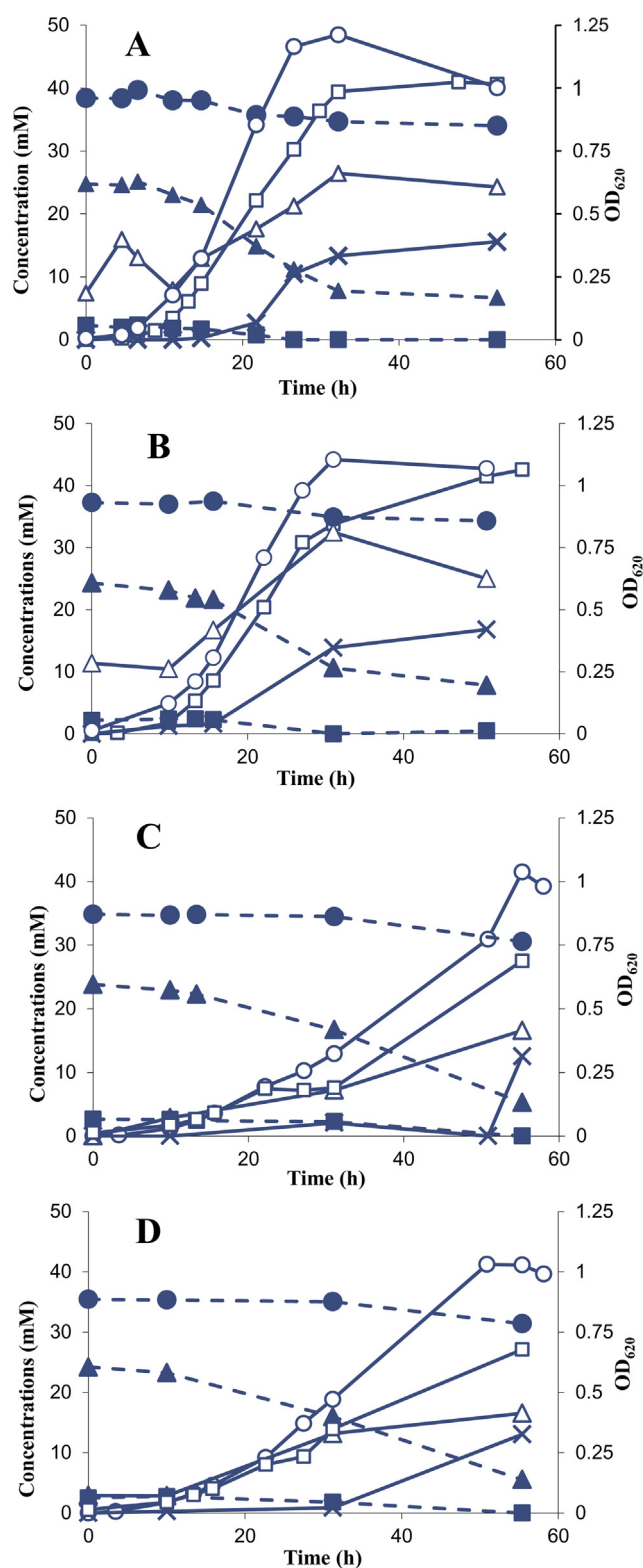
Fig. 2 — Batch fermentation profile of *C. saccharolyticus* cultures performed in closed serum flasks (Substrate, atmosphere in the headspace). WSH, N_2 (A), WSH, CH_4 (B), Pure sugars (glucose, xylose and arabinose), N_2 (C) and pure sugars, CH_4 (D). Glucose (●), xylose (▲), arabinose (■), OD_{620} (○), H_2 accumulation (□), lactate (✖) and Acetate (△). Each experiment is a representative of at least two independent replicates.

Table 1 − Results of the continuous fermentations of wheat straw hydrolysate by C. saccharolyticus.

Parameter	Results obtained at HRT (day) and at a sparging condition of:			
	0.83 (N_2) Case I	0.28 (N_2) Case II	0.83 ($N_2 + CO_2$) Case III	0.28 ($N_2 + CO_2$) Case IV
Biomass conc. (g/L)	1.25	1.07	0.47	0.54
(Q_{H_2})[b](L H2/L/day)	5.09	5.19	2.04	3.75
(q_{H_2})[c](L H2/g/day)	4.1	4.9	4.4	7.0
qsugar[d](g/g/day)[a]	5.3, 3.1, 0.3	8.4, 8, 0.8	3.7, 5.8, 0.8	7.5, 8.6, 0.8
Residual sugar[a](g/L)	0.9, 0.3, 0	3.9, 1.1, 0.05	5, 1.3, 0	5.4, 2.3, 0.2
Product yield (mol/mol)				
H$_2$	3.43	2.08	3.16	3.04
Acetate	1.69	1.07	1.75	1.66
Lactate	0.03	0.58	0.03	0.03
Ethanol	0.07	0.09	0	0.19
Conversion H2/total sugar (%)	88	46.3	33.4	19.2
Inhibitor reduction (%)				
HMF	32	5	16	20
Furfural	62	75	100	85
Osmolality[e]	0.23	0.21	0.25	0.25
Carbon recovery (%)	110	115	105	116
Redox recovery (%)	104	108	101	109

a Three values for three sugars, i.e. glucose, xylose and arabinose respectively.
b (Q_{H_2}), volumetric hydrogen productivity.
c (q_{H_2}), specific hydrogen productivity.
d q_{sugar}, specific sugar consumption rate.
e Osmolality was measured in Osmol/kgH$_2$O.

contained low concentrations of COD (<1 g/L) and volatile fatty acids (<0.1 g/L). Furthermore, the COD of the medium fed to the UASB reactor was reduced by approx. 95% after the treatment. Addition of modified anaerobic medium resulted in a need of a high reactor buffer capacity, which was reflected in the partial alkalinity that ranged from 5.4 to 5.8 g/L. The concentration of the buffer species NH_4^+−N, in the reactor varied from 0.66 to 0.74 g/L as a consequence of urea mineralization (Table 2).

Table 2 − Treatment of dark fermentation effluent in a UASB reactor.

Parameter	HRT (day)	
	2.5	1.5
Duration (days)	29	20
pH of influent	6.9	−
Influent COD	15.3	15.3
NH$_4$ − N (g/L)	0.12	0.12
OLR[a] (gCOD/L/day)	6.0 ± 0.5	10.5 ± 1.2
MPR[b] (l CH$_4$/L/day)	1.64 ± 0.12	2.64 ± 0.04
Methane yield (l CH$_4$/g COD)	0.28 ± 0.03	0.26 ± 0.04
Methane content (%)	60 ± 1	61 ± 4
pH of effluent	7.5	7.53
Effluent COD (g/l)	0.79 ± 0.05	0.78 ± 0.03
COD reduction (%)	95	94
Volatile fatty acids (g/l)	<0.01	0.06 ± 0.03
Partial alkalinity (g/l)	5.8 ± 0.2	5.4 ± 0.1
NH$_4$ − N (g/L)	0.74 ± 0.02	0.66 ± 0.11

a OLR, organic loading rate.
b MPR, methane production rate.

3.5. Overall energy output

On average, about 50% of the energy in wheat straw has been retrieved across all the scenarios of the *hythane* process. The energy output from DF was highest for *Case I* and lowest for *Case IV*. Although, the composition of effluent generated during different *Cases* of DF was different, due to the mixing of all the effluent together before its treatment, a scenario-specific energy output could not be determined for AD. Hence, a maximum energy output observed during AD was assumed to be true in all the scenarios of *hythane* (Table 3), which was significantly higher than the energy output from any of the DF *Cases* (Table 3). About 85% of the overall energy present in straw is contained in the sugars, of which 60% (average of all *hythane* scenarios, Table 3) has been successfully retrieved in the form of H$_2$ and CH$_4$ in the present *hythane* process.

4. Discussion

4.1. Dark fermentation

In this study, *C. saccharolyticus* was successfully cultured on WSH, provided that the concentration of WSH is less than 20% (v/v). *C. saccharolyticus* has been seen previously to grow efficiently on hydrolysates of wheat straw and Miscanthus, juices of sweet sorghum and sugar beet as well as on raw feedstocks, such as, maize leaves, *Silphium trifoliatum* leaves, potato peels, carrot pulp and paper sludge [34−39]. *C. saccharolyticus* has been observed to sustain growth in a medium containing up to 2 g/L of common growth inhibitors found in WSH, viz., 5-

Table 3 – Energy output in all scenarios compared with reference scenario. Values for energy contained in wheat straw (19.1 kJ/g) and in its sugar fraction (16.3 kJ/g) were obtained from Kaparaju et al. [3] and Nkemka et al. [27] respectively.					
Scenario:	Case I + AD	Case II + AD	Case III + AD	Case IV + AD	Case V[c]
Energy output (kJ/g straw)					
H_2 Production (LCV[a])	2.3	0.8	0.9	0.6	–
CH_4 Production (LCV)[b]	8.6	8.6	8.6	8.6	11.6
Total	10.9	9.4	9.5	9.2	11.6
Energy yield (%)					
LCV Products/energy in straw	57	49	50	48	61
LCV Products/energy in sugars	67	58	58	56	71

a LCV, lower calorific values.

b Since the effluent collected from different Cases of DF was mixed before its treatment in AD, the energy output for the latter was assumed constant in all the scenarios in this study.

c A reference case scenario wherein WSH was directly fed to an AD reactor [27].

(hydroxymethyl)furfural and/or furfural [34]. However, the concentrations of these inhibitors in the WSH used in this study were far below 2 g/L [27]. On the other hand, the osmolality of the medium containing 20% WSH was found to be about 0.26 Osmol/kg of H_2O, which is well above the critical osmolality, i.e. 0.22 Osmol/kg of H_2O, reported for substantial growth inhibition in a growing culture of C. saccharolyticus [23]. Hence, the inability of C. saccharolyticus to initiate growth on higher concentrated WSH is related to its limited osmo-tolerance.

The results herein revealed that C. saccharolyticus is as unaffected by CH_4 as by N_2. To our knowledge no information is available in the literature about the ability of thermophiles like C. saccharolyticus to grow in the presence of CH_4. Performance on WSH (10% v/v) was slightly better than on artificial medium, which might be due to the presence of marginal amounts of soluble proteins and amino acids in WSH [8,9,27]. No obvious explanation could be found for the observed slight beneficiary effect of the presence of CH_4 compared to N_2 (Figs. 1(B) and 2). Nevertheless, it strongly suggests that sparging with upgraded CH_4 can be an appropriate alternative. However, to obtain purified CH_4, CO_2 should be removed from the flue gas of the AD reactor, which will incur significant additional costs. To reduce these costs, the DF reactor can be sparged with the non-upgraded flue gas of the AD reactor i.e. mixture of CH_4 and CO_2. In addition, C. saccharolyticus can sustain growth in non-sparging conditions in the reactor [22], which opens an opportunity to alleviate the costs of sparging. However, H_2 yields obtained in the absence of sparging are much lower due to formation of more undesirable by-products such as lactic acid, which is also not a preferred substrate for acetoclastic methanogenesis in AD [40,41]. Hence, absence of sparging in the DF reactor can affect both DF and AD. A thorough techno-economic evaluation of the entire process may conclude the best applicable alternative.

The maximum overall H_2 productivities observed in the hythane scenario (Case I, Table 1) is at least five times higher than the average H_2 productivity reported by Kongjan et al. [9]. Moreover, the productivities observed in all the Cases in this study are comparable to previously reported values for C. saccharolyticus, ranging from 2.3 to 9.7 L of H_2/L/day, the highest of which was achieved when hydrolysed potato steam peels were used as a substrate [14,34–38]. The observation of significantly lower H_2 yield in Case II may have been due to

overflow metabolism, i.e. high glycolytic flux causing a metabolic shift at the pyruvate node to lactate formation. Overall, the combination of low biomass, volumetric H_2 productivity and sugar conversion efficiency of cultures sparged with $N_2 + CO_2$ clearly illustrate the dramatic effect of CO_2 in the sparging gas (Case III and IV, Table 1). A previous investigation on the effect of sparging with CO_2 in C. saccharolyticus cultures [23], revealed that the inherent formation of bicarbonate increased the osmotic potential to critical levels. As a consequence, extensive cell lysis occurs in the culture resulting in higher protein and DNA concentration in the culture broth [23]. Nevertheless, this nutrient-rich lysate might benefit the growth of the remaining cells, therefore displaying higher specific H_2 production rates observed in cultures sparged with CO_2 (Case III and IV, Table 1). Alternatively, the observation of CO_2 stimulating growth of C. saccharolyticus on xylose [42] might have improved specific H_2 productivity in Case III and IV.

None of the Cases studied showed complete consumption of sugars which could indicate a limitation of an essential nutrient. It can be argued that it might be sulphur. Firstly, phosphoric acid (H_3PO_4), instead of sulphuric acid (H_2SO_4), was used in the mild acid pretreatment of wheat straw used in this study, thus eliminating a potential sulphur source from the medium [27]. Secondly, the influents of all DF cases were supplemented with yeast extract as the only sulphur source. With a minimal concentration of 1 g/L it may not have provided adequate amounts of sulfur. Finally, wheat straw itself contains very negligible amounts of sulfur [43]. However, further experiments are needed to explore this hypothesis as they were out of the scope of this study.

The higher carbon and electron (redox) recovery observed in all the cases may have been due to traces of non-hydrolyzed disaccharides and/or oligosaccharides in WSH. This also may have resulted in a possible overestimation of H_2 yields in the respective cases.

4.2. Anaerobic digestion of the effluent collected from DF

The maximum methane production rate obtained during anaerobic digestion of the DF effluent collected from a H_2 producing CSTR during this study is significantly higher than a previously reported value (2.1 L CH_4/L/day) in a similar study where DF effluent was collected from a H_2 producing UASB

reactor [9]. This might be related to the differences in composition of DF effluent, as: i) the DF effluent collected during this study contained mainly acetate whereas, its counterpart in the previous study contained significant amounts of butyrate, propionate and ethanol, along with acetate [9], and ii) acetoclastic methanogens take acetate as a substrate and rely on acetogens for the conversion of butyrate, propionate and ethanol to acetate [40,41]. In another study [27], WSH was directly fed to a methanogenic UASB reactor at an OLR of 10.2 g COD/L/day producing methane at a production rate (2.7 L CH_4/L/day) comparable with the one reported in the present study.

So far, sustained organic loading rates up to 15 g COD/L/day have been reported in the treatment of DF effluents in a UASB reactor [3,9,44,45]. However, applications of OLRs higher than 15 g COD/L/day were observed to result in accumulation of volatile fatty acids, low COD reductions and low CH_4 yields. In addition, very high OLRs generate vigorous gas production rates, thus inflicting instability to the granular bed and eventually leading to process failure [45]. Due to a decrease in methane yield and slight increase in VFA accumulation at higher OLR (10.2 g COD/L/day, Table 2) further increase in OLR was abandoned in this study.

A stable pH within the range of 7−8 has been reported as optimum for acetoclastic methanogenesis [9]. Consumption of VFA during AD may have contributed to a pH increase to a suitable range.

Granular anaerobic sludge is known to be more protective for methanogens against inhibitory compounds than liquid granular sludge [46]. This could be a reason why batch tests of AD using liquid anaerobic sludge resulted in lower CH_4 yields on DF effluent (∼0.22 L CH_4/g COD) than obtained from effluent treated in the UASB reactor with granular anaerobic sludge (Table 2).

4.3. Overall energy output and the potential of the process

The overall energy yield obtained during this study (average of all *hythane* scenarios), i.e. approximately 2010 kJ/L of WSH, was about four times higher than the stable overall energy yield reported earlier for a similar study (440 kJ/L of WSH, estimated from Ref. [9]). Thus, in comparison, this study reports a very efficient process with respect to overall energy output. However, in the study performed by Kongjan et al. [9], the total sugar concentration in the culture medium was about twice lower than in this study, which resulted in comparatively lower H_2 and CH_4 yields per litre of WSH and consequently a lower energy yield.

Another study on biohydrogen production from WSH reports an energy yield of 0.96 kJ/g of wheat straw (estimated from Refs. [3,8]) which is two-folds lower than the energy yields obtained in *Case I* (Table 3) of the DF phase studied herein. In the present study, the overall conversion efficiency for a *hythane* process i.e. 60% could not match the high conversion efficiency i.e. 71% obtained in a study pertaining to production of biogas using WSH (Table 3 [27],). However, the former will be advantageous, if the aim is to produce *hythane*.

About 85% of the energy in wheat straw can be retrieved in the form of soluble sugars (Table 3). Although, reasonably high substrate conversion efficiencies can be achieved during DF and AD using the soluble sugars in WSH; the possible losses of sugars during the extensive pre-treatment process can result in much lower overall energy yields (Tables 1−3). Hence, an efficient pre-treatment process is of paramount importance for any *hythane*-like process.

In the current study, the AD expending about five-folds more process time than DF (1.5 days for AD and 0.28 days for DF), will consequently require reactors with five-folds more volumetric capacity than DF. Reactors with higher volumetric capacity will incur higher capital and operational costs. This can be conveniently avoided simply by operating DF reactors at high HRT (preferably similar to that of AD), which may also aid in achieving higher conversion during DF (Table 1 and 3).

Overall, the process offers a number of benefits with respect to convenience in operation and cost, i) a thermophilic DF process offers less risk of contamination by H_2-oxidising methanogens in the DF reactor [47], ii) the contaminants can also be kept out of the DF reactor by operating it at relatively higher growth rate [8] and iii) the process can successfully retrieve about 57% of the energy present in wheat straw. More technical details of the process and possible ways of cost reduction have been extensively discussed elsewhere [48].

5. Conclusions

C. saccharolyticus can efficiently produce H_2 from sugars in WSH. The residual sugars and acids produced can subsequently be converted to CH_4 in a methanogenic UASB reactor. The two-step process gives reasonable conversion efficiencies (about 67% of energy in the sugar fraction of wheat straw), but there remains room for further improvement. Moreover, the performance of *C. saccharolyticus* is not affected by CH_4 allowing application of this gas for sparging the hydrogenogenic reactor. However, a further extensive techno-economic evaluation is required to determine the best DF set up out of the following scenarios: i) sparging with upgraded CH_4, ii) sparging with the non-upgraded flue gas from the AD reactor, or iii) no sparging. An optimized and economically feasible version of this process can potentially complement a bio-refinery, wherein, along with bio-energy other value-added products are also produced from any unutilized parts of renewable agricultural biomass. This study paves a way for further exploration to determine whether a biological *hythane* process can be a viable alternative for the conversion of lignocellulosic biomass.

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