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

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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.
is more than evident that the world needs alternative, renewable energy sources which should also be environmentally 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 $4.7 \times 10^{14}$ GJ of energy (about 5% 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-sacharides 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 CH₄ 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 complemented 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 CH₄ or N₂. 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, H₂ accumulation and metabolite concentrations. Experiments were continued until H₂ 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) NH₄Cl 0.9 g, MgCl₂.7H₂O 0.4 g, KH₂PO₄ 0.75 g, K₂HPO₄ 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% N₂ or a gas mixture containing N₂ + CO₂ (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), N₂ sparging; Case II, higher growth rate (D = 0.15 h⁻¹), N₂ sparging; Case III, low growth rate (D = 0.05 h⁻¹), sparging with a mixture of N₂ (60% v/v) and CO₂ (40% v/v); and Case IV, higher growth rate (D = 0.15 h⁻¹), sparging with a mixture of N₂ (60% v/v) and CO₂ (40% v/v). The steady states were determined after at least five volume changes based on the stability of CO₂ and H₂ 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 CO₂ and H₂ 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, NH₄−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 H₂ productivity (mM/h) was calculated using the ideal gas law and the H₂ and CO₂ 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 H₂ and CO₂. 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 H₂ or CH₄ produced. The LCV for H₂ and CH₄ 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 H₂ yields. (Fig. 1(A)). Even though, the differences observed were insignificant, yet a decreasing trend can be observed in maximum obtainable H₂ productivities with increasing WSH concentration (Fig. 1(A)). Hardly any or no significant growth and H₂ accumulation was observed in the flasks containing 20% WSH (data not shown). Interestingly, H₂ 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.
the gas mixture of N₂ + CO₂ was assumed to mimic the non-upgraded flue gas (CH₄ + CO₂) from the AD (Case III and IV). Similarly, cultures sparged with N₂ were assumed to be the same as if sparged with CH₄ (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 h⁻¹) and sparging the reactor with N₂ resulted in the highest H₂ yield and best of substrate conversions (Table 1). The substrate conversion efficiency decreased with increasing growth rate and when CO₂ was present in the sparging gas. Surprisingly, at a higher growth rate (D = 0.15 h⁻¹), the culture sparged with N₂ + CO₂ displayed a higher H₂ yield and higher specific H₂ production rate than the one sparged with N₂ (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 H₂ productivity was about 40% higher in the reactors sparged with N₂ only (Table 1, 5.1 L H₂/L/day) than the reactors sparged with N₂ + CO₂ (Table 1, 2.9 L H₂/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 N₂ + CO₂ displayed higher medium osmolalities than their counterparts performed with N₂ sparging (Table 1). Similarly, low amounts of biomass were obtained in chemostats sparged with N₂ + CO₂ 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 N₂ + CO₂ (Case III and IV, Table 1), whereas the opposite was true for the cultures sparged with N₂ (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, N₂ (A), WSH, CH₄ (B), Pure sugars (glucose, xylose and arabinose), N₂ (C) and pure sugars, CH₄ (D). Glucose (○), xylose (△), arabinose (●), OD₆₂₀ (□), H₂ accumulation (▲), lactate (○) and Acetate (●). Each experiment is a representative of at least two independent replicates.
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 NH₄⁺, in the reactor varied from 0.66 to 0.74 g/L as a consequence of urea mineralization (Table 2).

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 H₂ and CH₄ 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-

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

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Results obtained at HRT (day) and at a sparging condition of:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.83 (N₂) Case I</td>
</tr>
<tr>
<td>Biomass conc. (g/L)</td>
<td>1.25</td>
</tr>
<tr>
<td>(Q_H₂) volumetric hydrogen productivity (L H₂/L/day)</td>
<td>5.09</td>
</tr>
<tr>
<td>(q_sugar) specific hydrogen productivity (g g⁻¹/d)</td>
<td>4.1</td>
</tr>
<tr>
<td>q_sugar (g g⁻¹/d)</td>
<td>5.3, 3.1, 0.3</td>
</tr>
<tr>
<td>Residual sugar (g/L)</td>
<td>0.9, 0.3, 0</td>
</tr>
<tr>
<td>Product yield (mol/mol)</td>
<td></td>
</tr>
<tr>
<td>H₂</td>
<td>3.43</td>
</tr>
<tr>
<td>Acetate</td>
<td>1.69</td>
</tr>
<tr>
<td>Lactate</td>
<td>0.03</td>
</tr>
<tr>
<td>Ethanol</td>
<td>0.07</td>
</tr>
<tr>
<td>Conversion H₂/total sugar (%)</td>
<td>88</td>
</tr>
<tr>
<td>Inhibitor reduction (%)</td>
<td></td>
</tr>
<tr>
<td>HMF</td>
<td>32</td>
</tr>
<tr>
<td>Furfural</td>
<td>62</td>
</tr>
<tr>
<td>Osmolality (°Osmol/kg H₂O)</td>
<td>0.23</td>
</tr>
<tr>
<td>Carbon recovery (%)</td>
<td>110</td>
</tr>
<tr>
<td>Redox recovery (%)</td>
<td>104</td>
</tr>
</tbody>
</table>

a Three values for three sugars, i.e. glucose, xylose and arabinose respectively. 
b (Q_H₂), volumetric hydrogen productivity. 
c (q_sugar), specific hydrogen productivity. 
d q_sugar, specific sugar consumption rate. 
e Osmolality was measured in Osmol/kg H₂O.

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

<table>
<thead>
<tr>
<th>Parameter</th>
<th>HRT (day)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2.5</td>
</tr>
<tr>
<td>Duration (days)</td>
<td>29</td>
</tr>
<tr>
<td>pH of influent</td>
<td>6.9</td>
</tr>
<tr>
<td>Influent COD</td>
<td>15.3</td>
</tr>
<tr>
<td>NH₄⁻ – N (g/L)</td>
<td>0.12</td>
</tr>
<tr>
<td>OLR (g COD/L/day)</td>
<td>6.0 ± 0.5</td>
</tr>
<tr>
<td>MPR (L CH₄/L/day)</td>
<td>1.64 ± 0.12</td>
</tr>
<tr>
<td>Methane yield (L CH₄/g COD)</td>
<td>0.28 ± 0.03</td>
</tr>
<tr>
<td>Methane content (%)</td>
<td>60 ± 1</td>
</tr>
<tr>
<td>pH of effluent</td>
<td>7.5</td>
</tr>
<tr>
<td>Effluent COD (g/L)</td>
<td>0.79 ± 0.05</td>
</tr>
<tr>
<td>COD reduction (%)</td>
<td>95</td>
</tr>
<tr>
<td>Volatile fatty acids (g/L)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Partial alkalinity (g/L)</td>
<td>5.8 ± 0.2</td>
</tr>
<tr>
<td>NH₄⁻ – N (g/L)</td>
<td>0.74 ± 0.02</td>
</tr>
</tbody>
</table>

a OLR, organic loading rate. 
b MPR, methane production rate.
The results herein revealed that C. saccharolyticus is unaffected by CH₄ as by N₂. To our knowledge no information is available in the literature about the ability of thermophiles like C. saccharolyticus to grow in the presence of CH₄. 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 CH₄ compared to N₂ (Figs. 1B and 2). Nevertheless, it strongly suggests that sparging with upgraded CH₄ can be an appropriate alternative. However, to obtain purified CH₄ CO₂ 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 CH₄ and CO₂. 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, H₂ 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 H₂ productivities observed in the hythane scenario (Case 1, Table 1) is at least five times higher than the average H₂ productivity reported by Kongjian 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 H₂/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 H₂ 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 H₂ productivity and sugar conversion efficiency of cultures sparged with N₂ + CO₂ clearly illustrate the dramatic effect of CO₂ in the sparging gas (Case III and IV, Table 1). A previous investigation on the effect of sparging with CO₂ 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 H₂ production rates observed in cultures sparged with CO₂ (Case III and IV, Table 1). Alternatively, the observation of CO₂ stimulating growth of C. saccharolyticus on xylose [42] might have improved specific H₂ 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 (H₃PO₄), instead of sulphuric acid (H₂SO₄), 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 H₂ 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 H₂ producing CSTR during this study is significantly higher than a previously reported value (2.1 L CH₄/L/day) in a similar study where DF effluent was collected from a H₂ producing UASB process.
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 [9,40,41]. 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 CH4/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 CH4 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 CH4 yields on DF effluent (~0.22L CH4/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 hynthane 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 H2 and CH4 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 1 (Table 3) of the DF phase studied herein. In the present study, the overall conversion efficiency for a hynthane process i.e. 66% 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 hynthane.

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 hynthane-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 H2-oxi-dising 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 H2 from sugars in WSH. The residual sugars and acids produced can subsequently be converted to CH4 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 CH4 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 CH4, 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 hynthane process can be a viable alternative for the conversion of lignocellulosic biomass.

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