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Hydrothermal hydrolysis of starch with CO_2 and detoxification of the hydrolysates with activated carbon for bio-hydrogen fermentation.

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Abstract

The imminent use of hydrogen as an energy vector establishes the need for sustainable production technologies based on renewable resources. Starch is an abundant renewable resource suitable for bio-hydrogen generation. It was hypothesised that starch hydrolysates from a large (250 mL) hydrothermal reactor could support bioH_2 fermentation without inhibition by toxic byproducts.

Starch was hydrolysed at high concentrations (40-200 g.L^{-1}) in hot compressed water (HCW) with CO_2 at 30 bar in a 250 mL reactor, the largest so far for polysaccharide hydrolysis, at 180-235 °C, 15 min. Hydrolysates were detoxified with activated carbon (AC) and tested in biohydrogen fermentations. The maximum yield of glucose was 548 g.kg starch^{-1} carbon at 200 °C. 5-hydroxymethyl furfural, the main fermentation inhibitor, was removed by AC to support 70% more hydrogen production than the untreated hydrolysates. The potential utilization of starch hydrolysates from HCW treatment for upscaled fermentations is promising.

Keywords:
Hot Compressed Water, Hydrothermal hydrolysis, Detoxification, Biohydrogen, Starch.

1. Introduction

The depletion of fossil fuel sources along with the greenhouse effect caused by increasing atmospheric CO_2 is driving the need for new clean energy alternatives to substitute for petroleum and meet increasing energy demand.

Hydrogen is an environmentally benign energy carrier that can be effectively utilized for power generation and it will play an important role in future energy technologies [1,2]. .

H_2 is a valuable commodity, its main uses being in the chemical industry, petroleum refining, ammonia production and as rocket fuel with a total world annual consumption of more than 50 million tonnes, with a market value of $120 billion in 2010 and 15% annual growth [2-4]. The potential use of H_2 as a major power source for stationary applications and for the transportation industry will considerably increase its demand [1].

Currently, strong investment in infrastructure consisting of the production, storage, transportation and the strategic deployment of H_2 refuelling station networks is taking place in countries like Japan, Germany, USA, China, U.K. and Canada among others. [5,6].

The majority of the world's supply of H_2 comes from fossil fuels which is not sustainable. In light of this scenario new production technologies not based on fossil fuel to generate H_2 are required.

Biological H_2 production by the fermentation of agricultural products, by-products and organic wastes (biomass) is a sustainable low-carbon technology for H_2 production. This technology is based on the capabilities of various microorganisms to evolve H_2 from sustainable organic materials.

Biomass is an abundant renewable resource capable of supporting the future H_2 economy [2,4,7]. In recent studies made by the Biomass R&D Technical Advisory Committee (BTAC) of the US Department of Energy and Agriculture [8] it is reported that biomass now exceeds hydropower as the largest potential domestic source of renewable energy. It currently provides over 3% of the total energy consumption in the United States where the total annual consumption of biomass feedstock for bioenergy and bioproducts together currently approaches 190 million dry tons. This study also found that the combined forest and agriculture land resources (1.3 billion tonnes) have the potential to supply sustainably more than 30% of the US current petroleum consumption for fuels and chemicals.

Starch, a main constituent of biomass, is one of the most abundant renewable organic compounds on Earth, being present in a wide variety of agricultural and staple food wastes such as potatoes, corn, rice, wheat and pasta. Starch comprises 1,4-α-linked glucosyl units in the form of linear, water insoluble amylose (20-25%) and 1,6-α-linked branched, water soluble amylopectin (75-80%).

Strict anaerobes like the clostridia and thermatogales can utilise starch directly. However, the demand on the cells to perform enzymatic hydrolysis limits the rate of H_2 production. By analysing the data of four reviews [9-12] and excluding duplicates, it was determined that fermentations using simple sugars produced H_2 with 3-fold higher specific rates (mmol H_2 [g DW.h]$^{-1}$) than fermentations using complex sugars. The analysis included 19 reports using complex sugars (starch and cellulose) and 27 reports using simple sugars (glucose, sucrose) with mean values of 4.8 and 14.6 mmol H_2 [g DW.h]$^{-1}$, respectively (t-test, P value: 0.030).

Hence, for complex polysaccharides (i.e. sustainable starch resources) to support bio-H_2 production, a pre-fermentation hydrolysis may be advantageous over an in-fermentation hydrolysis.

Hydrolysis can be achieved by several methods including chemical hydrolysis, enzymatic hydrolysis and hydrothermal hydrolysis. Enzymatic hydrolysis of starch is currently the preferred method in industrial use with high hydrolysis yields and mild conditions, although it incurs the costs of enzyme production. Thermostable α-amylases are highly attractive with high optimal temperatures (60-100 °C) and associated high reactivity [13] but this temperature range (and hence the potential reactivity) is well below that of hydrothermal hydrolysis (see below) while the α-limit dextrin of amylopectin is inaccessible without the concerted action of a debranching enzyme. Chemical hydrolysis leads to environmental and equipment corrosion problems as well as the costs associated with concentrated acids and post-hydrolysis neutralisation [14,15]. In contrast, hydrothermal hydrolysis is an environmentally benign method that, recently, has been the object of extensive research since the process only requires water and heat [16,17], which could be obtained internally in a waste-to-hydrogen process or externally from other renewable resources.

Hydrothermal hydrolysis using hot compressed water (HCW) is considered an alternative option to thermophilic enzymatic hydrolysis. At high temperatures (180-240 °C) the starch granules swell and burst, the semi-crystalline structure is lost and hydrolysis proceeds. Under these conditions, water possesses very interesting and unique properties which make it a powerful solvent suitable for the solvolysis of complex polysaccharides [18]. It has been demonstrated that at least 93% of starch is converted to soluble products at temperatures ranging from 180 to 220 ^{o}C under various conditions [17,19]

However, at temperatures above 200 °C the degradation of sugars to other compounds, mainly 5-hydroxymethylfurfural (5-HMF) and furfural, is unavoidable. These compounds, when present in the hydrolysate, are potent inhibitors of growth and fermentation and, therefore, should be removed [20]. Activated carbon (AC) has been tested as an effective approach for this purpose [21-23]. This is also attractive since AC can be derived from biomass char, a low value by product of the thermochemical conversion of biomass by pyrolysis.

Starch may be contained in various biomasses and the conditions of hydrolysis require optimisation specific to each biomass (depending mainly on its composition) to maximise sugar yields at the lowest possible temperature, to minimise heating cost and sugar decomposition.

This study investigated hydrolysis in HCW in the presence of CO_2. The addition of CO_2 into HCW hydrolysis was shown to enhance the yield of sugars from starch [24] and to reduce the concentrations of organic acids (fermentation inhibitors) in lignocellulose hydrolysates [25]. This effect was attributed to the action of CO_2 as an acid catalyst [26] unlike traditional acids CO_2 is extremely benign as it can be largely neutralised by the release of reactor pressure [25].

Table 1 summarises the five previous studies on starch hydrolysis in HCW, with and without CO_2, showing that previous reactors had very small volumes (3-80 ml). This small volume enabled the precise control of reaction conditions with rapid heating and cooling through bath techniques, which would not be efficient at large scale because of limited heat transfer.

Rapid heating and cooling is advantageous as it minimises the degradation of monosaccharides and associated formation of degradation products (furans, phenolics and organic acids). Sugar degradation occurs rapidly at temperatures above 100 °C particularly in the presence of acids or amines [29], whereas starch hydrolysis occurs above 200 °C [19]. Therefore, cooling time should be minimised because very little starch hydrolysis occurs in this phase, whereas the degradation of formed sugars continues.

The practicality of rapid heating and cooling decreases with increasing reactor size and the reactors required for practical application will be much larger than those studied to date (Table 1). Therefore, the fermentability of hydrolysates is a direct consequence of reactor size. For practical application, the technique must be scaled up and the consequences for practical process control require assessment. This study investigates the byproduct formation and fermentability of starch hydrolysates using a larger HCW/CO_2 reactor than any reported previously (250 ml) and unlike previous studies the results can be extrapolated to practical scale because of the non-bath type heating mechanism employed.

To evaluate the effectiveness of starch hydrolysis in HCW/CO_2 the fermentability of starch hydrolysates produced under a range of conditions, was assessed by the anaerobic fermentation of *E. coli* HD701 (an MC4100 derivative derepressed for formate hydrogenlyase) [30] as a convenient model organism to indicate fermentability.

The objective of this study was to test the effectiveness and viability of fermentable hydrolysate production from starch using hydrolysis in HCW/CO_2. Starch functions as a model system prior to extension of the approach to enable the effective use of lignocellulosic biomass as fermentation precursors. The hydrolysis of starch in HCW/CO_2 was examined at concentrations between 40-200 $g.L^{-1}$ in a 250 mL batch reactor which, as far as the authors are aware, is the largest reactor of this type reported for starch hydrolysis representing an important step towards scale-up of this technology. We also describe the consequences of scale-up for the practical control of reaction conditions, report the effects on product distribution and evaluate the product for its suitability as an *E. coli* fermentation substrate.

2. Materials and Methods

2.1. Materials.

All chemicals were analytical grade from (Sigma-Aldrich) and were used without purification. The activated carbon (AC) was colorsorb 5 steam activated powder from JACOBI (micropore, 0.19 $cm^3.g^{-1}$; mesopore, 0.37 $cm^3.g^{-1}$; macropore. 1.68 $cm^3.g^{-1}$; total surface area: 900 $m^2.g^{-1}$). *E. coli* HD701 was provided kindly by Prof. F. Sargent (University of Dundee).

2.2. Use of hot compressed water (HCW) for starch hydrolysis

The batch reactor system for starch hydrolysis is shown in Figure 1, comprising a 250 mL reactor (Parr series 4570/80 HP/HT) made of alloy C-276 and equipped with a heat/agitation controller (model 4836) and a cooling system (Grant LTD6/20). Temperature and pressure were measured from inside the reactor to within 1 bar and 0.1 K.

For hydrolysis, starch (5 g from potato powder) was suspended in de-ionized water to a final reactant volume of 125 mL (40 $g.L^{-1}$) or as otherwise stated and charged into the reactor for hydrolysis. This left a head space of about 120 mL. The reactor was sealed and purged with CO_2 (3 min) with agitation (850 rpm) before pressurising to 30 bar with CO_2 and heating to the set-point temperature. The reaction parameters are shown in Table 2. Reaction conditions were held for 15 min before cooling down to 100 °C by circulating water at 4 ºC through the reactor internal cooling loop (ID 0.8 cm) at a flow rate of 900 $cm^3.min^{-1}$. Next, the reactor was removed from the heating surround and quenched in an ice-water bath. The reactor was depressurized and the products were recovered by washing out with 20-40 mL of de-ionized water. The hydrolysate was separated from solid residue by vacuum filtration through two layers of filter paper (Fisherbrand QL100); hydrolysates and samples were kept at -20 ^oC for analysis. The residue was dried at 60 °C and weighed. It is important to note that after cooling some precipitate formed in some of the hydrolysates, this precipitate was removed by filtration and was not quantified.

Hydrolysate samples were analysed for organic acids (OA) by anion HPLC using a Dionex 600-series system [31] and sugars and 5-HMF by HPLC (Agilent 1100 series) equipped with on-line degasser, quaternary pump, auto-sampler and RI detector (1200 series). The column was a Resex-RCM (Phenomenex) equipped with a security column guard with the same stationary phase as the column. Sample injection was 20 µL; mobile phase: HPLC H_2O (Sigma); flow rate: 0.5 $mL.min^{-1}$; 40 min experiment time. Column temperature was 75 °C. RI detector was at 40 °C. The total organic carbon (TOC) of hydrolysates was measured using a TOC analyser (Model TOC 5050A, Shimadzu Co., Japan).

2.3 Detoxification

The hydrolysate was treated with 5% (w.v $aq.^{-1}$) AC powder (except where otherwise stated) at 60 ºC for 1 h with agitation at 180 rpm as described by Hodge *et al.* (2009) [21]. The treated hydrolysates were vacuum-filtered through filter paper (Fisherbrand QL100). Hydrolysates and samples were kept at -20 °C for tests and analysis.

2.4 Fermentation

The effectiveness of the AC treatment and the efficacy of the hydrolysates as fermentation feedstocks was evaluated. Small fermentation tests were performed using 60 mL glass serum bottles leaving 75% of volume for gas space and 25% for culture media (15 mL). The bottles were sealed (10 mm butyl rubber stoppers). The initial pH was standardised to pH 6.5 (± 0.1) with $NaOH/H_2SO_4$; additions for pH adjustment were negligible.

Stocks of *E. coli* HD701 were maintained at -80 ºC in 75% (w.v $aq.^{-1}$ glycerol), and revived by plating on nutrient agar (Oxoid) and incubating overnight (30 ºC). Colonies were picked from the plates into 5 mL vials of nutrient broth solution (Fluka) with added sodium formate (0.5% w.v $aq.^{-1}$) pH 7 (NBF8) and incubated for 6 h at 30 ºC, 180 rpm for pre-culture. Cultures (inocula 10 µL of sample equivalent to 0.001% inoculum) were grown in 2 L sterile Erlenmeyer flasks containing 1 L of the same medium; flasks were incubated at 30°C, 180 rpm, 16 h. Cell pellets were obtained by centrifugation (Beckman J2-21M/E centrifuge at 7500 rpm, 10 min, 20 °C), washed twice in 200 mL phosphate buffered saline (PBS: 1.43 g Na_2HPO_4, 0.2 g KH_2PO_4, 0.8 g NaCl, 0.2 g $KCl.L^{-1}$, pH 7.0) and then re-suspended in 25 mL of PBS with their concentration measured using a UV/visible spectrophotometer Ultrospec 3300 pro. The cells' concentration was estimated by optical density using a previously-determined conversion factor; OD_{600} 1 = 0.482 g dry weight.L^{-1}.

Reaction bottles for fermentation tests contained 10 mL of sterile medium consisting of Bis-Tris buffer (0.1 M) and Na_2SO_4 (0.0435 M; pH 6.5) and 5 mL of hydrolysate (filter sterilized) or glucose control (60 mM). Bottles were sealed using butyl rubber stoppers and made anaerobic by purging with N_2 for at least 30 min. 0.5-1 mL of the cell suspension was added to give a final cell concentration of 1 g dry weight.L^{-1}, before purging for a further 3-5 min.

Reaction bottles were incubated at 30 °C (180 rpm for 20 h). The identity of H_2 as the sole combustible gas present was confirmed using a ThermoQuest gas chromatograph (TraceGC2000) with a Shimadzu shincarbon-ST column and thermal conductivity detector. Routine measurement of H_2 concentration was performed using a combustible gas meter (Gasurveyor2, GMI), intermittently cross-validated by GC. Averages of 3 samples were converted using a linear calibration ($R^2 > 0.99$). The measured concentration of H_2 (y) was used to determine the volume of H_2 produced (x), using equation 1.

$$y = x/(ax+h) \therefore x = hy/(1-ay) \quad \text{(equation 1)}$$

Where:
y , [H_2] in headspace (v.v^{-1});
x , H_2 produced (mL);
h , headspace volume (mL)
a, the ratio of total gas produced to H_2 produced.

a was close to 2 in these tests as confirmed by GC. This is as expected from the known pathways of mixed acid fermentation in which H_2 and CO_2 arise exclusively from the cleavage of formate : HCOOH → H_2 + CO_2 [32]. Therefore H_2:CO_2 is initially 50:50; a=2. Two factors may influence a to be slightly different from 2. H_2 is oxidised by uptake hydrogenases but their influence is slight as shown by H_2 production tests of uptake hydrogenase-negative mutants [7] under conditions analogous to this study. There may also be a slight loss of CO_2 as part of the minor succinate formation pathway.

The variable addition of cell concentrate used in independent experiments and its minor effect on headspace volume was accounted for.

3. Results and Discussion

3.1. Hot Compressed Water (HCW) hydrolysis

Experiments were repeated twice and average values are reported. The worse case error was within 10%. Table 2 shows that the pH of the post-reaction solution (pH_f) decreased from the initial value of 7.0 (± 0.2) in most cases. The slight increase in pressure after the reactions may be an indication that some gasification of the products occurred (Table 2). Conversions of starch into hydrolysis products (X_h) were close to 100% in all cases, with a modest decrease with increasing temperature to a minimum value of 90% at 235 °C. Gas and residue were not analysed.

The incorporation of CO_2 in the HCW reactions enhances the yield of monosaccharides [24]. Each type of polysaccharide requires different optimal reaction conditions. Miyazawa reported a 14-fold increase in the glucose yield when solid CO_2 was included at a level of 9 g $CO_{2(s)}$.g $starch^{-1}$ for starch hydrolysis at 200 °C and 15 min [24]. In order to better simulate the functioning of a large scale system, the present apparatus used gaseous CO_2 and a maximum input of 1.4 g $CO_{2(g)}$.g $starch^{-1}$. In experiments carried out with cellulose (Orozco RL; unpublished) it was found that the addition of CO_2 at this level enhanced the yield of glucose ~1.5-fold compared to a N_2 control at 250 °C for 15 min (optimal parameters for hydrolysis of cellulose) and this level of CO_2 incorporation was adopted in the current study.

The heating and cooling rates have a strong influence on decomposition and product distribution. Larger reactors, such as the one used in this work, cannot achieve instant temperature changes. It is important, therefore, to study the product distribution under different conditions and realistic profiles of heating and cooling. Other studies on the hydrothermal degradation of polysaccharides (Table 1) used small batch reactors (total volume ~ 3.3-3.6 mL) heated rapidly from room temperature up to 200 °C by immersion in a molten salt bath maintained at constant temperature giving a heat-up period of about 2 min included in the reaction time for reaction completion. The reactor was quenched in cold water [19,27,33,34].

The heating and cooling profiles of the HCW system in this study are shown in Figure 2 with different set maximum temperatures; note that the reaction times including the heat-up (from 50 °C) and cool-down (to 50 °C) periods are within the range of 37-60 min, with corresponding average heating and cooling rates between 7-9 °C. min^{-1}. A hold time of 15 min at the set-point temperature was selected based on previous studies made on HCW hydrolysis of starch [17,24] and parallel studies here on cellulose using the same reactor system (Orozco RL; unpublished).

Figure 3 shows the product yield distribution with temperature after hydrolysis but before AC treatment. Glucose was the main product identified by HPLC in the hydrolysate followed by 5-HMF. Maltose and lower concentrations of fructose, mannose, galactose were observed. The yield of glucose increased with temperature, reaching its highest value (0.548 [g C. (g C in starting $starch)^{-1}$]) at 200 °C and then decreased, reaching minimal levels at 235 °C. The other sugars exhibited similar behaviour but 5-HMF the main inhibitory product, which results from the thermal degradation of sugars, reached its maximum yield (~ 0.3 [g C. (g C in starting $starch)^{-1}$] 30% carbon basis) at 220 °C.

Hydrolysates produced at 180 °C contained almost no sugars or 5-HMF but nevertheless had a TOC value similar to the maximum obtained, due to the presence of dextrins from partial starch hydrolysis, which was confirmed by Agilent HPLC (see Materials and Methods) with reference to a maltodextrins from potato starch standard (Sigma-Aldrich 419699; dextrose equivalent: 16.5-19.5). The relatively low fermentability observed for hydrolysates obtained at 180 °C (Figure 5) suggests that the majority of these short-chain polysaccharides were in branched forms, which (unlike linear maltodextrins) cannot be utilised by *E. coli* as it lacks a debranching enzyme [35].

The maximum sugar yield obtained (at 40 g $starch.L^{-1}$, 200 °C) was very similar to yields reported previously using smaller reactors (Table 1). Therefore, the level of CO_2 used in the present work was effective in promoting hydrolysis but may have also enhanced the formation of 5-HMF as reported in previous studies [24-26]. Based on these results, HCW hydrolysis of starch at concentrations of 120 and 200 $g.L^{-1}$ were performed at an optimal temperature of 200 °C.

In addition to sugars, 5-HMF and minor products, organic acids (OA), which are decomposition products of glucose and fructose, were found in the hydrolysate in very small concentrations at starch concentration of 40 $g.L^{-1}$ and 200 °C. Butyric acid and acetic acid were the main OA produced (Table 3). OA yields at higher concentrations of starch were negligible which indicates insignificant degradation of 5-HMF and furfural at 200 °C.

The removal of toxic hydrolysis products by the treatment of hydrolysates with AC proved to be very effective. The concentrations of all sugars were relatively unaffected by AC treatment whereas significant removal of 5-HMF (Figure 4) and OA (Table 3) was observed.

It is noteworthy that the yield of hydrolysis products in HCW can be affected by leaching of the reactor material or nickel alloy [36,37] however these were not evaluated.

TOC analysis before and after AC treatment (Table 4) indicated high extents of C removal, of which up to 85% was through the elimination of 5-HMF. This confirms that the pore structure of the AC used in this work was appropriate [12]. The 5-HMF retention capacity of the AC was in the range 0.033-0.096 g 5-HMF.[g AC]$^{-1}$. For comparison, 0.06-0.12 g [g AC]$^{-1}$ was reported previously [21]. Furfural, was found in preliminary work, to be at least 10-fold lower in concentration than 5-HMF, was removed by AC treatment and was not considered further.

3.2 Fermentation of starch hydrolysates and biohydrogen production

Figure 5 shows H_2 production and yields in fermentability tests using AC-treated and untreated hydrolysates from the HCW/CO_2 hydrolysis of starch (40 g.L^{-1}). AC treated hydrolysates showed higher H_2 production than their untreated counterparts which demonstrated the effectiveness of the AC treatment in the removal of inhibitors for *E. coli* HD701. Hydrolysates made at (180, 220 or 235 °C; AC treated) contained 1.5, 53 and 6 mM glucose respectively (diluted 1/3 in fermentability tests to 0.5, 17.7 and 2 mM; initial glucose concentration) and glucose was completely consumed in the cases where H_2 was produced (Figure 5). Hence, H_2 production was limited by both substrate availability and by the influence of degradation products (DPs).

However, the hydrolysate made at 200 °C (AC treated), contained 106 mM glucose (35.3 mM in fermentability test; an excess of 15.3 mM compared to glucose control), leaving residual unused glucose after H_2 production. The initial substrate concentration is not critical in *E. coli* fermentations but due to the limited buffering capacity of the test medium, pH limitation occurred when the initial glucose was in excess of ~ 20 mM. This arose using hydrolysates made using higher starch loadings during hydrolysis (Table 5) and, in these cases, H_2 production was affected only by DPs.

The removal of 5-HMF was complete when starch was hydrolysed at a concentration of 40 g.L^{-1} but at 120 g.L^{-1} and 200 g.L^{-1}, 5.2 mM and 15.9 mM 5-HMF persisted, respectively. As shown in Table 5, the hydrolysate glucose concentration increased with the initial starch loading but the H_2 yield decreased, suggesting inhibition by residual DPs. The hydrolysates were, therefore, diluted with deionised water to provide 20 mM glucose, leaving 0.33 mM 5-HMF and 0.6 mM 5-HMF, respectively. H_2 yields from diluted hydrolysates were indistinguishable from the glucose control and unrelated to the initial starch loading. Therefore, dilution minimised the impact of persistent inhibitory DPs in these hydrolysates and further AC treatment would be required at scale.

The maximum theoretical H_2 yield in *E. coli* is 2 mol H_2.mol hexose^{-1} [31]. Therefore, the observed yields represent about 19% (including the glucose control). The fermentability tests used here provided a rapid, high throughput screening of hydrolysates for the investigation of hydrolysis conditions, but provided sub-optimal fermentation conditions (inconstant pH, end product accumulation, fixed volume, poor mixing), to which the low conversions are attributed. The conversion of glucose to H_2 by *E. coli* in sophisticated fermentation systems is well described and yields of close to 100% have been independently reported [38, 39] and also observed by the authors [40,41].

A detailed evaluation of the energy demand of HCW hydrolysis, within a waste-to-hydrogen process, indicated that the energy requirement of HCW would be ~10-20% of the electrical energy recoverable from bio-H_2 production (Redwood, Orozco and Macaskie, unpublished); this will be reported in full with reference to real wastes in a subsequent publication.

5. Conclusions

Hot compressed water (HCW) with CO_2 is an effective and potentially scalable method for starch hydrolysis. Detoxified hydrolysates from a 250 ml scale system here utilising scalable components (electric furnace and gaseous CO_2) were equal in fermentability to pure glucose for *E. coli* HD701. Therefore, a production scale HCW/CO_2 system of similar design could be expected to support *E. coli* fermentations as a sustainable alternative to refined glucose.

The relatively large reactor used here (see Table 1) showed similar yields to smaller systems indicating that hydrolysis in HCW/CO_2 have the potential for large scale practical application. The optimum temperature for starch hydrolysis in HCW/CO_2 was 200 °C. Glucose was the main product with a yield of 548 g.kg^{-1} starch which is very similar to the previous reports (Table 1). This shows that the relatively low CO_2 levels employed here using methods applicable at scale were sufficient and not limiting to starch hydrolysis. The generation of 5-HMF, however, was about 4-fold higher than in the previous reports, possibly due to the presence of CO_2 and the longer heating and cooling times associated with larger reaction volumes. AC treatment was effective in the selective removal of 5-HMF with an adsorption capacity in the range of 33-96 [mg 5-HMF (g AC)$^{-1}$]. Under the selected conditions up to 86% of the carbon removed from the hydrolysate by AC was attributable to 5-HMF loss and typically furfural (representing up to 10%) was largely removed. As a consequence, fermentations of the treated hydrolysates produced more than 70% more H_2 than untreated controls at optimum HCW conditions. At temperatures above 200 °C, noteworthy H_2 production occurred only with AC treated hydrolysates (Figure 5) but further detoxification was required for hydrolysates after hydrolysis with more than 40 g.L^{-1} starch (Table 5).

The fermentability of hydrolysates (obtained at 40 g.L^{-1} starch, 200 °C) was the same as glucose controls. At higher initial starch concentrations it was possible to produce hydrolysates with glucose concentration of up to 536 mM but additional detoxification was required to produce H_2 at the same level as pure glucose. This demonstrates that *E. coli* could adapt well to lower concentrations of other inhibitors and that dilutions improved the fermentability of the hydrolysate. This finding has a significant impact for reactor system utilisation efficiency and productivity. Future work will aim to further up-scale HCW while retaining optimum bio-conversion of starch to H_2.

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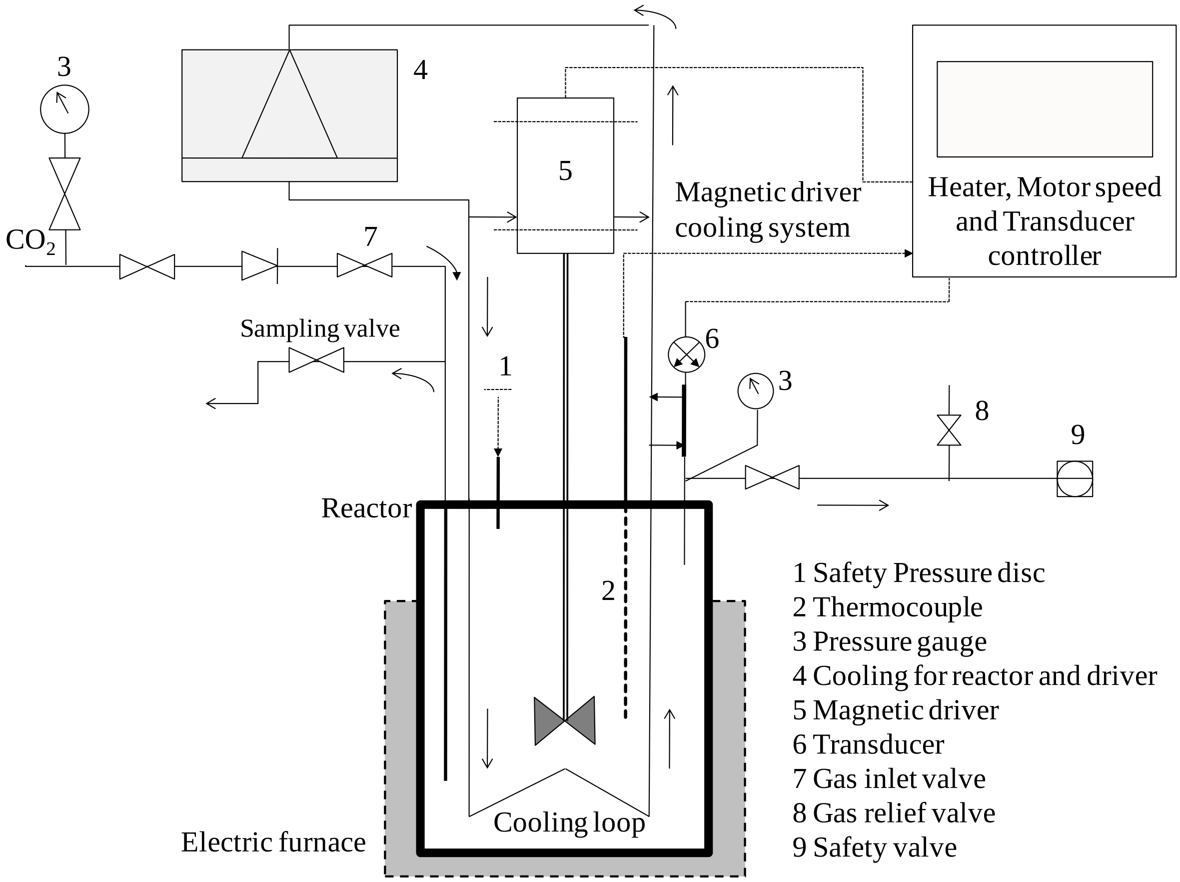

Figure 1. Batch reactor system for HCW hydrolysis. Arrows show direction of flow of CO_2, coolant or sample.

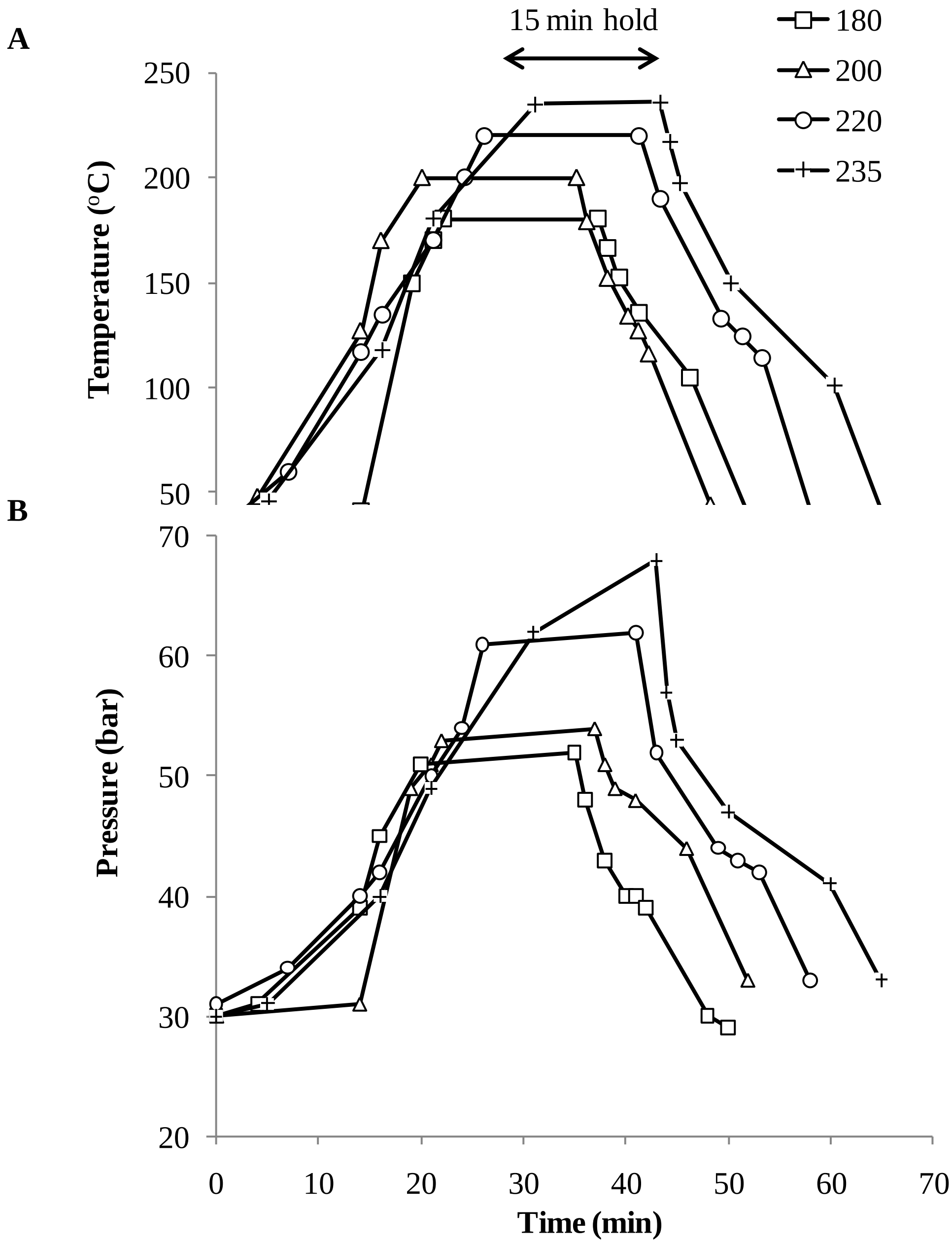

Figure 2. a) Reaction heating and cooling pathways; b) Pressure pathways at corresponding temperatures: (—□—) 180 °C, (—△—) 200 °C, (—○—) 220 °C and (—+—) 235 °C. 1 bar = 100 kPa. Reactions were performed in sequence from the lowest to the highest temperature. As a result, the temperature of the electric heating jacket was lower in the first reaction (180 °C) compared to the following reactions, resulting in ~5 min delay to reach 40 °C for the first reaction. Above 40 °C, the heating rate was consistent among experiments. Therefore, this delay was not considered to impact on the final products.

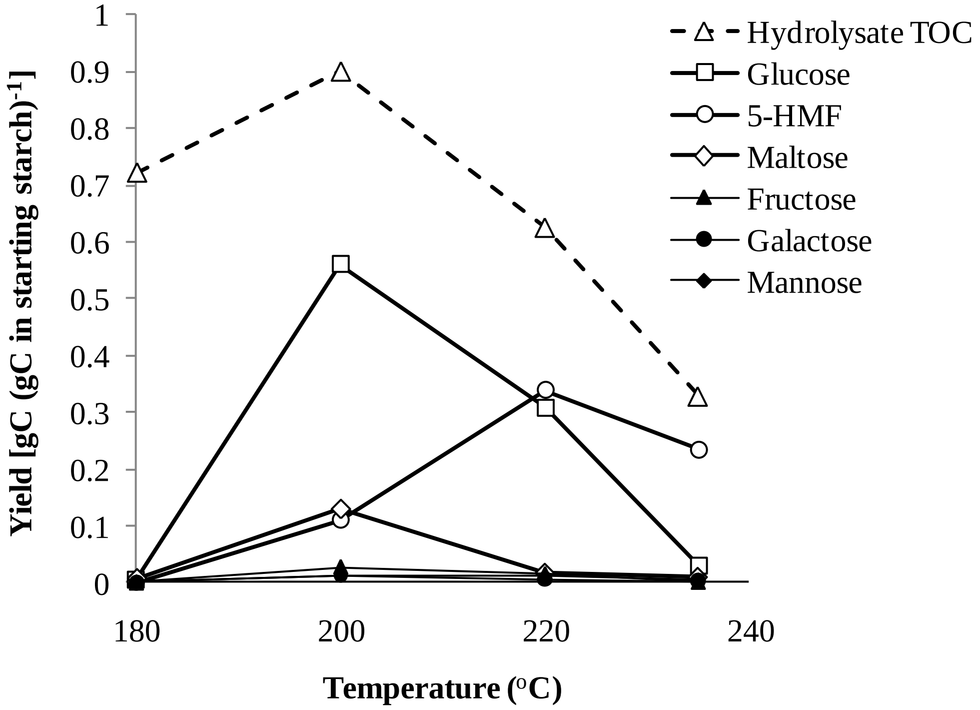

Figure 3. Product yield vs temperature before AC treatment. Hydrolysate values were calculated as a ratio of individual TOC in hydrolysate vs. TOC in starting starch. Symbols: (- △ -) hydrolysate TOC; (—□—) glucose; (—◇—) maltose; (—○—) 5-HMF; (—▲—) fructose; (—●—) galactose; (—◆—) mannose.

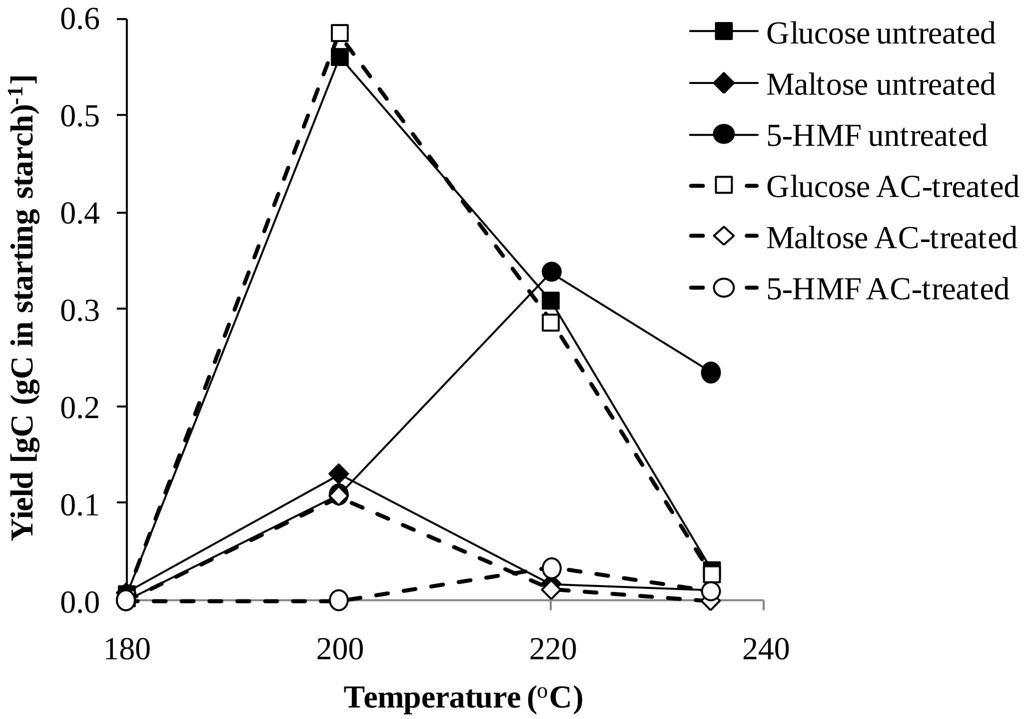

Figure 4. Product yield of main sugars in hydrolysates before (solid lines) and after (dashed lines) AC treatment. Values reported are mean of 2 experiments, variation was within 10%.

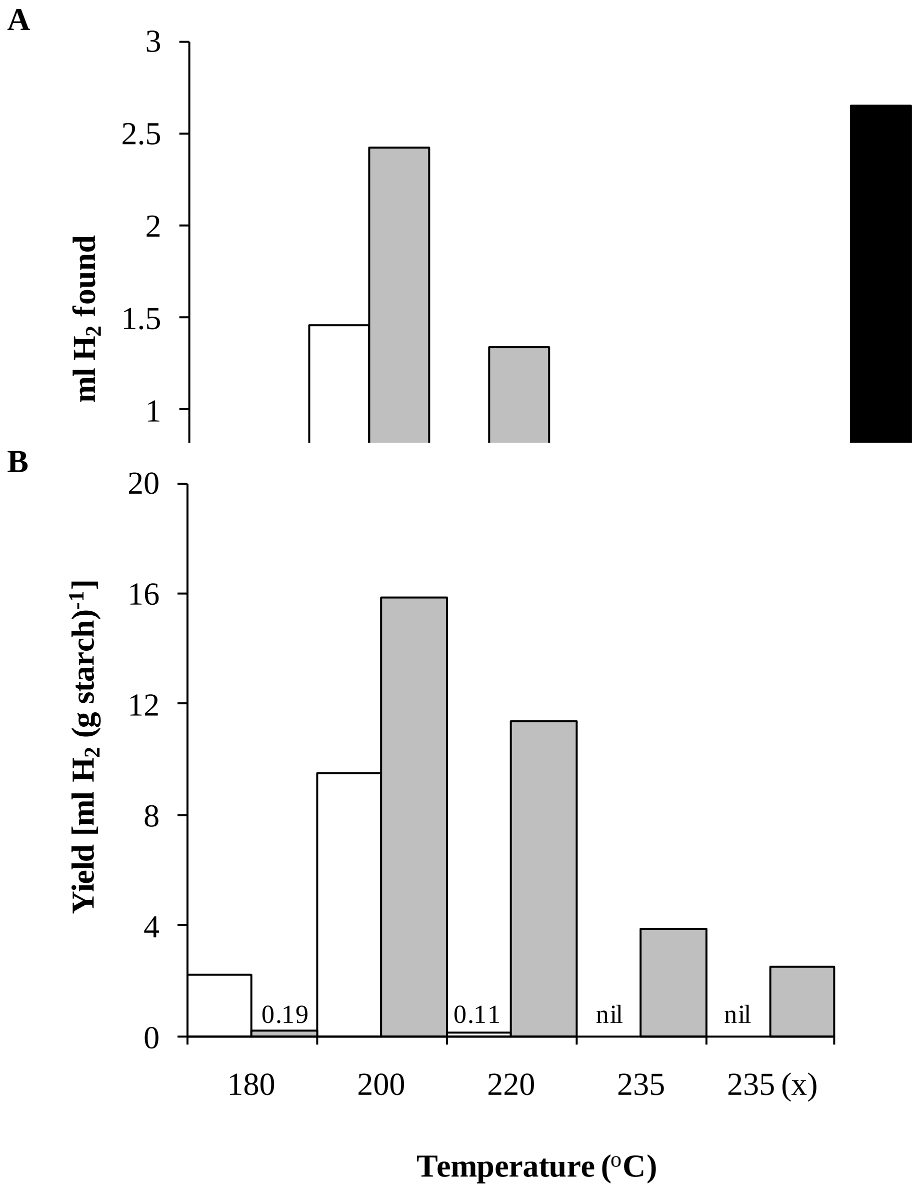

Figure 5. H_2 production from hydrolysate vs. hydrolysis temperature and AC treatment in small fermentability tests; a) mL H_2; b) yield [mL H_2 (g starch)$^{-1}$]. White bars represent hydrolysates before AC treatment; grey bars represent values after AC treatment (5% w.v aq^{-1}) except 235 °C (x), where 7.5% AC was used instead of 5% in an attempt to remove more inhibitors and improve H_2 production. The black bar represents pure glucose (20 mM in fermentability test) as a positive control. . Data represent the mean of 2 experiments, variation was within 11%.

Table 1. Previous studies on starch hydrolysis in hot compressed water with carbon dioxide (HCW/CO_2)

Reactor volume (type)	**CO_2**	**Heating method**	**Yield (% C to sugar)**	**Source**
3.3 ml (batch)	No	Molten salt bath	~63 %	[17]
3.6 ml (batch)	Yes	Molten salt bath	~53 %	[24]
33.28 ml (batch)	No	Molten salt bath	~43.8 %	[27]
80 ml (batch)	Yes	Sand bath	NA	[25]
Pipe, 3 mm ID (continuous)	Yes	Water bath	NA	[28]
250 ml (batch)	Yes	Electrical furnace.	~55 %	This study

Table 2. Reaction parameters for hydrolysis of starch at various concentrations

Initial starch concentration	**Temp** (°C)	**P_f** (bar)	**pH_f**	**X_h** %	**Glucose yield** g C (g C in starting starch)$^{-1}$
40 g/L	180	34	3.7	99.9	0.002
	200	32	3.2	99.8	0.548
	220	33	2.8	97.8	0.287
	235	31	3.0	90.4	0.027
120 g/L	200	32	2.8	99.3	0.608
200 g/L	200	33	2.8	99.3	0.628

Initial pH was 7.0 (± 0.2) and initial pressure was 30 (± 1) bar for all experiments. P_f: final pressure; pH_f: final pH; X_h: Starch conversion into soluble hydrolysis products. X_h was calculated according to: $X_h = (S - R)/S$ where S = Initial starch (g); R = Residue after the reaction (g).

Table 3. Yield of organic acids from starch hydrolysis

[Starch]	**40 g.L^{-1}**	
Organic acid	**BAC**	**AAC**
Lactic	0.0013	0.0006
Acetic	0.0041	0.0021
Formic	0.0019	0.0007
Butyric	0.0063	0.0014

Organic acid (OA) yields in [g C. (g C in starting starch)$^{-1}$] in starch hydrolysates obtained at 40 g starch.L^{-1} at 200 °C before (BAC) and after (AAC) activated carbon (AC) treatment (5% w.v aq.$^{-1}$). OA yields at starch concentrations of 120 and 200 g.L^{-1} were negligible.

Table 4. Removal of 5-HMF from hydrolysates by activated carbon.

Starch in hydrolysis	**Maximum Temp**	**AC loading**	**Before AC**		**After AC**		**5-HMF removed**	
			TOC	**5-HMF**	**TOC**	**5-HMF**		% of
$g.L^{-1}$	^{o}C	$g.L^{-1}$	$g.L^{-1}$	mM	$g.L^{-1}$	mM	$g\ (g\ AC)^{-1}$	removed C
40	180	50	8.9	0.0	3.2	0.0	0.000	0.0
	200	50	11.1	13.0	7.6	0.0	0.033	26.8
	220	50	7.7	40.5	4.6	4.0	0.092	84.7
	235	50	4.1	28.1	1.2	1.2	0.068	68.1
	235	100	4.1	28.1	0.5	0.0	0.035	56.8
120	200	50	34	42.3	30.9	5.2	0.093	86.1
200	200	75	55	73.0	43.4	15.9	0.096	35.4

Before AC, TOC values represent the carbon that remained soluble in the hydrolysate, which included 5-HMF; after AC, 5-HMF and other organic compounds such as organic acids and sugars (less than 5 %) were removed affecting the TOC content. The last two columns show the mass of 5-HMF removed mass of AC used in the treatment and as a % of the removed carbon from the hydrolysate.

Table 5. Effective HCW hydrolysis conditions, glucose yields and H_2 production.

Starch in hydrolysis ($g.L^{-1}$)	**Undiluted hydrolysates**			**Diluted hydrolysates**		
	Glucose in fermentability test (mM)		Yield (mol H_2/mol glucose consumed)	Glucose in fermentability test (mM)		Yield (mol H_2/mol glucose consumed)
	Start	End		Start	End	
40	35.0	12.4	0.29	20	0	0.35
120	104.0	86.0	0.17	20	0	0.37
200	179.0	159.9	0.14	20	0	0.38
Glucose control[a]				20	0	0.35

Conditions producing glucose concentrations ≥ 60 mM after hydrolysis at 200 °C are shown. Other sugars were minor after treatment at 200 °C. Glucose concentrations in detoxified hydrolysates were 3-fold higher than initial glucose concentrations in fermentabilty tests (Start) as 5 mL hydrolysate was added to 10 mL medium. Fermentability tests were done in triplicate and yields varied within ± 4% (means are shown).