Alcoholic fermentation of thermochemical and biological hydrolysates derived from *Miscanthus* biomass by *Clostridium acetobutylicum* ATCC 824

Mahendra P. Raut[a], Trong K. Pham[a], Leonardo D. Gomez[b], Ioanna Dimitriou[c] and Phillip C. Wright[d] *

Author affiliation:

[a] Department of Chemical and Biological Engineering, The University of Sheffield, Mappin Street, Sheffield, S1 3JD, UK

[b] CNAP, Biology Department, University of York, Heslington, York, YO10 5DD, UK

[c] Department of Chemical and Environmental Engineering, The University of Nottingham, University Park, Nottingham, NG7 2RD, UK

[d] School of Chemical Engineering and Advanced Materials, c/- Faculty of Science, Agriculture and Engineering, Devonshire Building, Newcastle University Newcastle upon Tyne, NE1 7RU, UK

*Corresponding author information: E-mail: phillip.wright@newcastle.ac.uk Ph: +44 (191) 208 4960

Abstract

This laboratory scale study aims to demonstrate the effectiveness of thermochemical and biological saccharification of *Miscanthus giganteus* (MG) for generation of fermentable saccharides and its subsequent fermentation into solvents i.e. acetone, ethanol and butanol (ABE) using *Clostridium acetobutylicum* ATCC 824. Saccharide hydrolysates were derived from MG by thermochemical (water, acid and alkali at 130 ^{o}C) and biological saccharification (*Fibrobacter succinogenes* S85) processes and were subjected to batch fermentation for 120 hours using *C. acetobutylicum* ATCC 824. At the end of fermentation of thermochemically-derived hydrolysates, 742 g m^{-3} of saccharides from water treatment, 9572 g m^{-3} of saccharides from acid treatment and 4054 g m^{-3} of saccharides from alkali treatment were fermented and yielded 0.045, 0.0069 and 0.01 g g^{-1} of total solvents, respectively. Similarly, at the end of fermentation of biological hydrolysate (using *F. succinogenes*), 2504 g m^{-3} of saccharides was fermented and yielded 0.091 g g^{-1} of total solvents. The highest yield of total solvents was achieved by water (thermochemical) and biological saccharification of MG using *C. acetobutylicum*. Whereas, acid and alkali-treated hydrolysates showed lower yields of solvents presumably due to production of inhibitory compounds during saccharification. Compared to thermochemical saccharification, biological saccharification using *F. succinogenes* is a promising approach since it yielded the highest amount of solvents whilst being eco-friendly. Our future studies will focus on optimisation of biological saccharification (using *F. succinogenes*) and sequential co-culture fermentation (using *C. acetobutylicum*). The development of alternative consolidated bioprocessing approach using biological saccharification will contribute towards making lignocellulosic biofuels a reality.

Keywords: Thermochemical saccharification; Biological saccharification; *Miscanthus giganteus*; *Clostridium acetobutylicum* ATCC 824; *Fibrobacter succinogenes* S85; ABE fermentation

1. Introduction

Biofuel production from lignocellulosic materials (wood, agricultural and forest residues) is a sustainable alternative to existing fossil fuels. Lignocellulosic biomass has a unique place in future biofuel production that can provide both, sustainable and eco-friendly alternative fuels [1].

Lignocellulosic biofuel production involves two main steps: 1) deconstruction of cell wall polymers in lignocellulosic biomass into saccharides *via* pre-treatment and saccharification and 2) conversion of those saccharides into biofuels *via* fermentation. However, the major bottleneck in lignocellulosic biomass to biofuel conversion is the recalcitrant nature of lignocellulosic polymers that makes the saccharification step rate limiting [2].

In order to bring lignocellulosic biomass into hydrolysates containing fermentable saccharides and also to make it more amenable for microbial fermentation, various physical, chemical and biological saccharification techniques has been employed [3-5]. Conventional physical and chemical saccharification techniques, including liquid hot water, steam explosion, CO_2 explosion, ozonolysis, solvents and acid/alkali processes, have been in use for biomass deconstruction [6], but require significant energy inputs or/and the addition of chemicals. For instance, liquid hot water treatment requires high amount of water and elevated temperature (170–230°C) and

pressure (up to 5 MPa), stream explosion requires high-pressure saturated steam (0.69–4.83 MPa) and high temperature (160–260°C), CO_2 explosion requires extremely high pressure, and ozonolysis, solvents and acid/alkali treatments requires addition of chemicals [7, 8]. Most of these techniques generate by-products that have inhibitory effect to subsequent fermentation processes. Conversely, biological saccharification is an ideal option due to lower energy input, but it is slow and less efficient [3]. Therefore, at present neither of these techniques are fully optimised, and still requires rigorous research to obtain cost effective and efficient pre-treatment for saccharification and robust subsequent fermentation method.

Clostridium acetobutylicum ATCC 824 (hereafter referred to as *C. acetobutylicum*) is an industrially important model microbe that produces acetone, ethanol and butanol (ABE), as well as hydrogen from various saccharides, which makes it suitable to ferment different agricultural and industrial wastes. Since *C. acetobutylicum* is unable to hydrolyse lignocellulosic polymers (cellulose and hemicellulose) directly, it is necessary to bring fermentable saccharides into hydrolysates by either chemical or biological pre-treatment and subsequent saccharification [9, 10].

1.1. Chemical strategy: thermochemical saccharification and fermentation

Ideally, acid/alkali pre-treatments of biomass at high temperature generate hydrolysates containing high amounts of fermentable saccharides [11] that can be further converted into fuels by fermentation. Clostridial species are well equipped to produce solvents using their multi-substrate utilising capacity more efficiently than any other genus of the three domains (Bacteria, Archaea, Eukaryota) [12]. In particular, *C. acetobutylicum* and *Clostridium beijerinckii* are good producers of solvents in acetone-butanol-ethanol fermentation (ABE), with the potential to ferment

a wide-range of saccharides in hydrolysates derived from agriculture residues [12]. ABE fermentation of different typical feedstocks hydrolysates using different strains of Clostridia are cited elsewhere [13].

1.2. Microbial strategy: biological saccharification and fermentation

Microbial strategies for saccharification, on the other hand, are diverse and represent a promising approach for the development of biological processes for industrial scale production of biofuels [14]. Consolidated bioprocessing (CBP) is an alternative microbial bioprocessing approach in which the key steps for lignocellulosic biofuel generation, i.e. saccharification and fermentation, occur simultaneously and employs combinations of natural and recombinant microorganisms [15]. Anaerobes with efficient lignocellulose degradation and biofuel generation capabilities are of particular interest [16]. The combination of microbes with desirable abilities such as saccharification and fermentation can provide a major breakthrough as an alternative CBP approach.

Thus considering the overall objective of CBP, sequential co-culture fermentation of lignocellulosic biomass is a viable solution over energy intensive thermochemical saccharification and fermentation methods. The CBP approach has been investigated by numerous research groups using Clostridia, however underperformance of lignocellulosic co-culture fermentation has been observed, and is attributed to a rather slow rate of hydrolysis [17]. A similar multi-organism approach was tested for bioenergy production from lignocellulosic biomass, using *C. acetobutylicum* and *Clostridium cellulolyticum* showing that the rate of lignocellulose utilization in the co-culture is improved compared to a *C. cellulolyticum* mono-culture

[18, 19]. *C. cellulolyticum and Rhodopseudomonas palustris* were also syntrophically grown as co-cultures. The increase in cellulose degradation observed by *C. cellulolyticum* was due to the removal of an inhibitory by-product (pyruvate) by *R. palustris* [20]. In a different study, *C. acetobutylicum* and *Ethanoigenens harbinense* were tested for biohydrogen production using microcrystalline cellulose as a substrate. Improved cellulose saccharification and hydrogen production were observed, compared to that of monoculture conditions [21].

1.3 Our approach

In this study, we attempted, for the first time, a sequential biological saccharification and fermentation approach with *F. succinogenes* S85 (hereafter referred to as *F. succinogenes*) and *C. acetobutylicum,* respectively. Among the selected anaerobic strains; *F. succinogenes* is the most efficient saccharolytic bacterium found in the herbivore rumen [22, 23], while *C. acetobutylicum* has significant capability to ferment a diverse range of saccharide components into ABE production [10, 12]. Our hypothesis was that combining *F. succinogenes* and *C. acetobutylicum* in a CBP approach will produce ABE solvents and hydrogen at a level comparable to those achieved using *C. acetobutylicum* fermentation of saccharides produced using conventional thermochemical saccharification strategies. To test our hypothesis, we compared production of ABE solvents and hydrogen between *C. acetobutylicum*-mediated fermentation of saccharides produced from lignocellulosic MG biomass using three thermochemical treatments (water/acid/alkali) and a CBP approach using a co-culture of *F. succinogenes* and *C. acetobutylicum* with three different substrates, acid-swollen cellulose (ASC), microcrystalline cellulose (MC) and lignocellulosic MG biomass.

2. Materials and methods

All chemicals used in this study were purchased from Sigma Aldrich (UK), unless otherwise indicated.

2.1. Microorganisms used and medium preparation

2.1.1. Clostridium acetobutylicum

C. acetobutylicum ATCC 824 was procured from the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany). *C. acetobutylicum* was grown anaerobically in a 125 cm^3 capacity serum bottle fitted with butyl rubber and crimp sealed containing 100 cm^3 media. The media composition was used as described by Lopez Contreras *et al*. [24] having the following composition per dm^3 (hereafter denoted as CA media): 0.75 g KH_2PO_4, 0.75 g K_2HPO_4, 0.348 g $MgSO_4$, 0.01 g $MnSO_4.H_2O$, 0.01 g $FeSO_4.7H_2O$, 1 g NaCl, 5 g yeast extract, 2 g $(NH_4)_2SO_4$, 1 g cysteine HCl (as reducing agent) and with 5 g glucose as a carbon source. The medium was heated to boiling and cooled down by flushing with nitrogen gas for 10 min. The bottles were crimped sealed with butyl rubber and autoclaved for 15 min at 121 ^oC. The medium was inoculated with a freshly-prepared inoculum and incubated at 37 ^oC for 18 to 20 hours (up to the exponential phase).

2.1.2. Fibrobacter succinogenes

F. succinogenes S85 (ATCC 19169) was kindly provided by Prof. Paul Weimer (US Dairy Forage Research Centre, Madison, Wisconsin, USA). *F. succinogenes* was cultivated under anaerobic conditions at 37 ^{o}C in a modified Dehority medium (MDM) as described by Weimer et al. [25, 26].

To prepare the basal media (FS media), the following stock solutions were each prepared first in a dm^3: a) mineral solution I; 22.5 g KH_2PO_4, b) mineral solution II; 11.26 g NaCl, 11.26 g $(NH_4)_2SO_4$, 1.06 g $MgCl_2.6H_2O$, 0.82 g $CaCl_2.2H_2O$, 0.344 g $MnCl_2.4H_2O$, 0.250 g $FeSO_4{\cdot}7H_2O$, 0.118 g $ZnCl_2$, and 0.026 g $CoCl_2.6H_2O$, 80 g Na_2CO_3, c) volatile fatty acid (VFA) solution; mixture of 1% (v/v) isobutyric acid, 1% (v/v) isovaleric acid, 1% (v/v) n-valeric acid and 1% (v/v) 2-methylbutyric acid), d) 8% Na_2CO_3 solution and e) reducing agent solution; 25 g cysteine HCl. Except mineral solution II, all stocks solutions (100 cm^3) were prepared by boiling and cooling whilst sparging continuously with nitrogen for 10 min in 125 cm^3 serum bottles, crimped sealed and autoclaved for 15 min at 121 ^{o}C. Schaefer's vitamin solution was also prepared as described by Callaway and Martin [25].

2.2. Basal medium (FS media)

Basal medium was prepared by adding 8 cm^3 of stock solution II into 79.5 cm^3 of distilled water, boiled and cooled whilst sparging with carbon dioxide for 10 min in a 125 cm^3 bottle, and autoclaved at 121 ^{o}C for 15 min. In an anaerobic chamber, to mixture, 4 cm^3 of mineral solution I, 3 cm^3 of VFA solution, 4 cm^3 of 8% Na_2CO_3 solution, 4 cm^3 of reducing agent and 0.1 cm^3 of Schaefer's vitamin solution were added. The final composition of the basal medium was (per dm^3): 0.9 g KH_2PO_4, 0.9 g NaCl, 0.9 g (NH_4) $2SO_4$, 0.084 g $MgCl_2{\cdot}6H_2O$, 0.065 g $CaCl_2{\cdot}2H_2O$, 0.027 g

$MnCl_2 \cdot 4H_2O$, 0.02 g $FeSO_4 \cdot 7H_2O$, 0.009 g $ZnCl_2$, and 0.0048 g $CoCl_2 \cdot 6H_2O$, 3.2 g Na_2CO_3, 0.06% (v/v) each of isobutyric acid, isovaleric acid, n-valeric acid and 2-methylbutyric acid, 1 g cysteine·HCl and 0.1% (v/v) Schaefer's vitamin solution.

2.3. Development of syntrophic co-culture media

Since both bacteria require different culture media for optimal growth, it was necessary to optimise the media in such a way that both bacteria can grow in the same medium. To obtain the modified co-culture media, 6 media bottles of each FS and CA media were prepared with 5 g dm^{-3} glucose as a carbon source. Both the media were then combined to obtain the ratio (FS:CA) of 100:0, 20:40, 40:60, 60:40, 80:20 and 0:100. Two sets of these combinations were prepared anaerobically in pre-sterilized 125 cm^3 serum bottles caped with butyl rubber and crimp sealed. These modified media were then inoculated with *F. succinogenes* (OD_{675} =0.72) and *C. acetobutylicum* (OD_{600} = 1.2). The growth of *F. succinogenes* and *C. acetobutylicum* were monitored by measuring optical density (OD) at wavelengths of 675nm and 600nm respectively. The growth profiles of *F. succinogenes* and *C. acetobutylicum* at different combination of FS and CA media are shown in Appendix A. Supplementary data **Fig. S1**. The mixed culture growth of both bacteria in the modified co-culture medium was imaged using an Olympus microscope BX51 (Tokyo, Japan) fitted with a CapturePro 2.6-JENOPTIK Laser camera (Optik, System GmbH, Germany). Finally the ratio of 40:60 (FS:CA) was selected as modified syntrophic co-culture medium for saccharification and fermentation.

2.4. Preparation of MG hydrolysate and fermentation

MG was grown in York, North Yorkshire, UK, under field conditions. The materials used represent the sixth year of harvest. After harvest and drying, it was milled using a Restch impact mill to 1 mm particles. The composition of raw MG is cellulose (34% ± 2.5 %), hemicellulose (42% ± 2.8 %), lignin (28% ± 2%) and ash (0.83% ± 0.03%). MG hydrolysate was obtained by treatment with either hot water or 100 mol m^{-3} H_2SO_4 or 200 mol m^{-3} NaOH at 130°C for 40 min. The supplementary salt medium was added to each bottle containing hydrolysates at concentration suggested by Wang and Chen [21]. The supplementary salt medium contained (per dm^3): 6 g $(NH4)_2SO_4$, 1.77 g KH_2PO_4, 2.938 g K_2HPO_4, 2 g $CaCO_3$ and 10 mg p-aminobenzoic acid, 10 mg biotin and 1 cm^3 mineral salt solution as described by George *et al.* [27].

The hydrolysates were then neutralised to pH 6.5 (optimal pH for growth and acid production) using H_2SO_4 and NaOH and centrifuged at 1000 x g for 2 min to remove precipitates. Supernatants obtained from each treatment were then sterilised using 0.2 µm polyethersulfone steritop-GP Millipore filter (Loughborough Fisher Scientific UK). A total of 400 cm^3 of MG hydrolysate from each treatment (biological triplicates) was added to 500 cm^3 capacity bottles fitted with rubber tight caps provided with inlet and outlet ports. The hydrolysates were further boiled and cooled down by continuous flushing with nitrogen for 10 min. Finally, bottles were tightened using clips. A reducing agent cysteine-HCl (1 g dm^{-3}) was added to remove remaining oxygen from the bottles. The pH of the media was finally re-checked to ensure that the pH was 6.5. The medium was inoculated with 4 cm^3 of freshly prepared inocula of *C. acetobutylicum* to each bottle and incubated at 37°C. The experimental set-up of the fermentation of MG hydrolysate is shown in Appendix A. Supplementary data **Fig. S2**. Finally, the supernatant collected from the fermentation broth and were

subjected to acetone, butanol, ethanol. The headspace gas was collected for hydrogen concentration measurements.

2.5. Biological saccharification of lignocellulosic biomass and fermentation

For biological saccharification, we selected MG, ASC, and MC. Alkali pre-treatment was employed on MG in order to remove maximum lignin from the biomass [28] and to get access to cellulose for biological saccharification using *F. succinogenes*.

One hundred cm^3 of this optimised syntrophic co-culture media (ratio of FS to CA = 40:60) was prepared with 5 g dm^{-3} of MG, ASC, and MC as a carbon source. Triplicate bottles of the media for each condition were firstly inoculated with *F. succinogenes* to achieve saccharification. *F. succinogenes* immediately adhered to the cellulose substrate particles and subsequently produced biofilms and released saccharide into the solution [29]. After inoculation, bottles were incubated at 37^{o}C for 40 hours (approximately 40 hours was required to achieve mid-exponential phase of growth on cellulose). During this period, to avoid utilisation of the released monosaccharides by planktonic cells and to achieve maximum saccharification, bottles were kept stagnant to allow biofilm formation. After 40 hours of incubation, the media was then inoculated with *C. acetobutylicum*. The sampling times were selected based on ethanol and butanol production in fermentation broth. As a result, supernatants were collected after 80 and 120 hours of incubation, analysed for ethanol, butanol and acetone, and the headspace gas analysed for hydrogen. Appendix A. Supplementary data **Fig. S3** shows *F. succinogenes* growth on MC cellulose and subsequent fermentation by *C. acetobutylicum*.

2.6. Dry weight of cellulosic biomass measurements

The final dry weight of MG, ASC and MC in fermentation broth were determined as described elsewhere [30]. Briefly, 15 cm^3 of broth was collected from bottles and centrifuged at 3000 *g* for 10 minutes, and then the substrate pellet was washed twice with 0.1% (w/v) methylcellulose solution to remove bound cells from the substrates. Substrate pellets were further washed with distilled water and centrifuged at 3000 *g* for 10 minutes. The supernatants were removed and tubes were dried in an oven (Nuve, EN 120) at 80^oC until a constant mass was reached. The difference in the final and initial weights of samples was assumed to be the substrate utilised by co-culture for biofuel production.

2.7. Analysis of saccharide concentration in MG hydrolysate derived by thermochemical treatment

The monosaccharides were separated by high performance anion-exchange liquid chromatography on a Dionex ICS-3000 using a Carbopac PA-20 column (Dionex, Camberley, UK) with integrated amperometry detection as described elsewhere [31]. The separated monosaccharides were quantified by using external calibrations with an equimolar mixture of four monosaccharides standards (arabinose, glucose, mannose and xylose). Each run takes 35 minutes with 25 minutes regeneration. The buffer system has two phases: 0.5 cm^3 min^{-1} flow in 1% (w/v) NaOH (200 mol m^{-3}), and then a mixture of 47.5% H_2O, 22.5% (w/v) NaOH (200 mol m^{-3}), and 30 % of

NaOH (100 mol m^{-3}) sodium acetate (500 mol m^{-3}). The chromatographic separation was developed at 30°C.

2.8. Analysis of fermentation products

Fermentation products were identified and quantified as previously reported by Pham *et al.* [32]. Briefly, acetone, ethanol and butanol, were detected and quantified using a GC- chromatograph Agilent 7890A (Cheshire, UK) system coupled with a 30 m × 0.25 mm ID × 0.25 µm Stabbilwax fused silica column (Thames Restek, UK). Approximately 50 mm^3 aliquots were collected, centrifuged at 17,000 *g* for 2 min and 2 mm^3 of sample was injected into the GC system. The GC was controlled and automated by ChemStation Agilent (Rev: 32.3.8) software. The total GC analysis running time was 14 min and temperature gradient was performed with a hold at 45°C for 3 min, followed by a ramp at a rate of 15°C min^{-1} to 120°C, then 30°C min^{-1} to 210°C and finally a hold 1 min at 210°C. Helium was used as the carrier gas at a flow rate of 1 cm^3 min^{-1}. The concentration of by-products ethanol, butanol and acetone were estimated by obtained standard curves for the respective metabolites based on its retention time and peak area. The injector, detector and oven temperatures were 250, 350 and 120°C respectively. A flame ionisation detector (FID) was used to detect and measure the by-products concentration. Products' (solvents) productivity was calculated as total solvents (present in the fermentation hydrolysate) produced in g m^{-3} divided by the fermentation time and is expressed as g m^{-3} h^{-1}. Solvents yield was calculated as total solvents produced divided by total saccharides utilized.

2.9. H_2 gas estimation

Gas samples were collected from the headspace of the sampling bottles using 10 cm^3 gas tight syringes at different interval times, depending on the sample types and sample was then injected in to a Varian CP-3800 gas chromatograph (Varian, Polo Alto, CA) equipped with a 500 mm^3 sample loop capacity. This volume was then directly injected *via* the Varian 1041 splitless on-column injector. Component separation was achieved using a Haysep (C18-100 mesh, porous polymer column, 2.0 m length and 0.32 cm inner diameter with 2 mm solid support) and a molecular sieve (13X, 60-80 mesh, packed column 1.5 m length, 0.32 cm inner diameter with 2 mm solid support) with argon carrier at a flow rate of 3.6 cm^3 min^{-1}. A Thermal Conductivity Detector (TCD) was used to detect hydrogen production. The GC was controlled and automated by the Star GC workstation (Version 5.50) software package (Varian). The instrument was calibrated using standard H_2 calibration gas supplied by BOC speciality gases (Guildford, Surrey, UK). An overview of the overall methodology is shown in **Fig. 1.**

Fig. 1 goes here

3. Results and discussion

Saccharification of lignocellulosic polymers is mandatory in order to ferment them into useful by-products, both in viewpoint of bioenergy and environment. The basic challenge for successive or simultaneous saccharification and fermentation of lignocellulosic polymers is to obtain high degree of hydrolysis for subsequent high

biofuel yield. Thus efforts for optimisation of efficient pre-treatments techniques will continue.

In this study, we employed thermochemical (water/acid/alkali pre-treatment at 130 ^{o}C) as well as biological (*F. succinogenes*) saccharification of MG to achieved fermentable saccharides into solution for subsequent fermentation by *C. acetobutylicum* (**Fig. 1**). We show that both thermochemical and biological saccharification of lignocellulosic biomass produced fermentable saccharides and these were subsequently fermented by *C. acetobutylicum*. Both thermochemical and biological saccharification/fermentation approaches produced ethanol, butanol and hydrogen. Interestingly, acetone production was below detection limit during fermentation. This observation is consistent with our previous study on synthetic hydrolysate (containing lignin) in which toxic effect on solvent production in *C. acetobutylicum* was observed [10]. In fact, several previous studies observed that the factors such as culture conditions, medium composition, substrates/products toxicity, reaction kinetics, enzymes and pH could influence dynamics of the ABE fermentation pathways in *C. acetobutylicum* [33-35]. Interestingly, study on ABE fermentation of hydrolysates derived from corncob [36] and domestic organic waste (DOW) [37] observed that the highest production of acids (called “Acid crash”) [38] resulted in premature cessation of ABE production ending-up with lower production of solvents.

3.1. Changes in saccharides concentration before and after thermochemical hydrolysates fermentation

In the first approach, saccharides (glucose, xylose, arabinose and mannose) obtained by saccharification of MG using H_2O, 100 mol m^{-3} H_2SO_4 and 200 mol m^{-3}

NaOH at 130^{o}C were subjected to fermentation by *C. acetobutylicum*. Glucose, xylose, arabinose and mannose were the major fermentable saccharides of the MG hydrolysates**.** The changes in concentration of saccharides before and after fermentation show active utilisation of the saccharides in fermentation as shown in **Table 1**.

Table 1 goes here

The concentrations of fermentable saccharides in the hydrolysate varied among the treatments. The highest concentrations of saccharides was produced in the hydrolysate derived by 100 mol m^{-3} H_2SO_4 treatment (607 g m^{-3} glucose, 6229 g m^{-3} xylose, 1627 g m^{-3} arabinose and 1399 g m^{-3} mannose), whereas the lowest saccharides concentrations were observed in hydrolysate derived by H_2O treatment (155 g m^{-3} glucose, 170 g m^{-3} xylose, 114 g m^{-3} arabinose and 311 g m^{-3} mannose). Xylose was the most abundant saccharide in the hydrolysates examined, particularly in acid treated hydrolysates. This is in agreement with previous observations that acid treatment efficiently degraded hemicelluloses, producing xylose [39, 40]. After fermentation, concentrations of these saccharides significantly reduced in all treatments (**Table 1**), which is in agreement with the previous study demonstrating that *C. acetobutylicum* can utilise a variety of saccharides including hexoses (e.g. glucose) and pentoses (D-xylose and L-arabinose) [12] to produce biofuels. The supporting information is provided in Appendix B. Supplementary data (XLSX).

3.2. Fermentation products from thermochemical hydrolysates fermentation

Significant reduction in saccharides concentration after fermentation clearly suggested that saccharides released into the hydrolysate solutions were used to produce fermentation by-products by *C. acetobutylicum* depending on amount of saccharides produced from each treatment condition. **Fig. 2A to C** show production of ethanol, butanol and H_2 in different thermochemical treatment conditions at 80 hours and 120 hours of incubation.

Fig. 2 goes here

Ethanol production (**Fig. 2A**) shows variation among the pre-treatments at 120 hours of fermentation. Ethanol production were relatively higher for 200 mol m^{-3} NaOH (40 g m^{-3} culture) and 100 mol m^{-3} H_2SO_4 (44 g m^{-3} culture) treatments compared to H_2O (34 g m^{-3} culture). The highest butanol production was observed for the H_2SO_4 treatment (19.7 g m^{-3} culture) compared to NaOH treatment (4.3 g m^{-3} culture), while no butanol production was observed in the H_2O treatment (**Fig. 2B**). The absence of butanol production in H_2O treatment and lower production of butanol in the 200 mol m^{-3} NaOH treatment might be a result of lower concentration of saccharides in the hydrolysates obtained by both these treatments (**Table 1**). The concentrations of saccharides were comparatively higher in the 100 mol m^{-3} H_2SO_4 treated hydrolysate and that was reflected in the higher concentrations of ethanol/butanol and H_2 produced (**Table 1**). This agrees with previous studies, where it was noted that saccharides concentration in hydrolysates affected subsequent biofuel production

and elevated level of glucose or saccharides in the medium resulted in induced butanol production [41].

Hydrogen, which is a clean and efficient replacement to fossil fuels, was also produced in all treatments. The highest production of H_2 was observed in H_2SO_4 treatment (0.081 mol m^{-3} of culture) while H_2O and NaOH treatments were lower, 0.035 mol m^{-3} of culture and 0.0084 mol m^{-3} of culture respectively. The lowest production of H_2 gas was found in the NaOH treatment condition possibly due to generation of soluble lignin and other inhibitor by-products by NaOH treatment that might affect H_2 production [42]. Our results suggested that the biomass treatment conditions significantly affected butanol, ethanol and H_2 productions. Overall, results showed that the H_2SO_4 treatment resulted in a higher yield of by-products (ethanol ; 44.4 g m^{-3}, butanol; 19.7 g m^{-3}, H_2; 0.081 mol m^{-3}) compared to H_2O (ethanol ; 34 g m^{-3} and H_2; 0.035 mol m^{-3}) and NaOH (ethanol ; 39.7 g m^{-3}, butanol; 4.2 g m^{-3}, H_2; 0.0084 mol m^{-3}) treatments. The production of fermentation by-products from hydrolysate by *C. acetobutylicum* is purely based on type of lignocellulosic biomass and pre-treatment conditions used [43]. The previous studies reported that 9600 g m^{-3} of total saccharides were fermented to 3400 g m^{-3} butanol, 500 g m^{-3} acetone, and 900 g m^{-3} ethanol [43].

3.3. Changes in lignocellulosic substrate concentration before and after biological hydrolysates fermentation

In the second approach, in order to grow *F. succinogenes* and *C. acetobutylicum* as a syntrophic co-culture, we modified the growth media (as mentioned in section 2.3) so that it could allow both these two bacteria to grow in a single fermentation vessel. The optimum growth performance for both bacteria was observed at a combination

of 40 % FS and 60 % CA media (modified syntrophic co-culture media) with growth rates of 0.074 h^{-1} (doubling time 9.36) and 0.179 h^{-1} (doubling time 3.85) for *F. succinogenes* and *C. acetobutylicum* respectively (Appendix A. Supplementary data **Fig. S1**). At this combination, the maximum OD_{675nm} for *F. succinogenes* and OD_{600nm} for *C. acetobutylicum* reached 0.912 and 1.018 at 30 hours of incubation respectively. The mixed culture growth of both bacteria in the modified co-culture medium is shown in **Fig. 3**. This modified co-culture medium (40 FS: 60 CA) was supplemented with 5 g dm^{-3} of each substrate ASC, MC and MG as a sole carbon source. In this study, we observed that *F. succinogenes* was able to hydrolyse cellulosic materials since 5 g dm^{-3} of each ASC, MC and MG were reduced to 1.77 ± 0.351 g dm^{-3}, 3.09 ± 0.433 g dm^{-3} and 2.5 ± 0.774 g dm^{-3} respectively.

Fig. 3 goes here

3.4. Fermentation products from biological hydrolysates fermentation

The production of ethanol, butanol and H_2 was observed in all cellulose substrate conditions. However, depending on the type of substrates, the concentration of products varied as shown in **Fig. 4 A to C**.

Fig. 4 goes here

Ethanol production was observed to be higher in ASC supplemented medium (241 g m^{-3}), compared to MC (211 g m^{-3}) and MG (217 g m^{-3}). A slight decrease in ethanol concentration were observed in ASC (241 g m^{-3} ± 36 g m^{-3} to 212 g m^{-3} ± 55 g m^{-3}) and MC (212 g m^{-3} ± 48 g m^{-3} to 198 g m^{-3} ± 43 g m^{-3}) hydrolysate between 80 hours between 120 hours fermentation mainly because of volatilization and ethanol condensation at top [44]. The maximum butanol productions were 11.2 g m^{-3}, 13.7 g m^{-3} and 13.2 g m^{-3} for ASC, MC and MG supplemented medium, respectively. A slight difference in butanol production was noted among these 3 substrate conditions. On the other hand, H_2 production reached the highest concentration in the ASC (0.03 mol m^{-3}) followed by MG (0.029 mol m^{-3}) and MC (0.007 mol m^{-3}). The higher productions of fermentation products were observed in the presence of ASC (ethanol; 241 g m^{-3}, butanol; 11.2 g m^{-3} and H_2; 0.03 mol m^{-3}) and MG (ethanol; 217 g m^{-3}, butanol; 13.2 g m^{-3} and H_2; 0.029 mol m^{-3}) than MC (ethanol; 211 g m^{-3}, butanol; 13.7 g m^{-3} and H_2; 0.008 mol m^{-3}). A possible reason for this is ASC and MG are pre-treated before saccharification thus combined pre-treatment and saccharification makes substrates more susceptible to microbial hydrolysis to release maximum fermentable saccharides [5] into the solution to produce more biofuels over MC. Our results suggest substrate dependent fermentation flexibility of *C. acetobutylicum*.

Previous studies reported syntrophic co-culture fermentation of cellulosic materials; eg. *C. cellulolyticum* and *R. palustris* produced 1243 g m^{-3} ethanol and 41mol m^{-3} H_2, [20], *C. acetobutylicum* X9 and *E. harbinense* B49 produced 55.4 mol m^{-3} H2 h^{-1} g^{-1} dry cell [21] and *C. thermocellum* JN4 and *Thermoanaerobacterium thermosaccharolyticum* GD17 produced 1.8 mol H_2 mol^{-1} of glucose [45]. In this study, we have shown for the first time that two efficient mesophilic lignocellulose

degrading/fermenting microbes, *F. succinogenes* and *C. acetobutylicum,* were able to grow syntrophically, producing C6 and C5 saccharides and converting them to ethanol, butanol and H_2 in a single fermentation unit as a CBP. No external enzymes or additives were required since cellulolytic/xylanolytic activity of *F. succinogenes* [10, 22] generated saccharides (C6 and C5) that *C. acetobutylicum* could utilise and produce biofuels *via* a fermentation process.

3.5. Comparison of total solvents yield and productivity achieved from both approaches

Total solvents yield and productivity during fermentation of hydrolysates (thermochemical and biological) derived from different lignocellulosic substrates were shown in **Table 2**.

Table 2 goes here

The total yield of solvents in thermochemically treated hydrolysates were 0.045 g g^{-1}, 0.0069 g g^{-1} and 0.01 g g^{-1} for MG hydrolysates treated with H_2O, 100 g m^{-3} H_2SO_4 and 200 g m^{-3} NaOH respectively. While total yield of solvents in biologically treated hydrolysates were 0.066 g g^{-1}, 0.103 g g^{-1} and 0.091 g g^{-1} derived from ASC, MC and MG substrates respectively. The total solvents yield and productivity of biologically derived hydrolysates were comparatively higher than thermochemically obtained hydrolysates **(Table 2)**. Previous studies showed much higher solvents yield and productivity than the present study [13]. The yield and productivity in previous studies using thermochemical saccharification approaches were between 0.30 to 0.40 g g^{-1} and 0.140 to 0.63 g dm^{-3} h^{-1} respectively [13]. However,

hydrolysates used in the previous studies were derived from different wastes (other than MG), and also were detoxified and supplemented with pure saccharides such as glucose and lactose. Moreover, all the strains of Clostridia used in previous studies were other than *C. acetobutylicum* ATCC 824. Thus, it is difficult to make a direct correlation with this study. The highest yield 0.1 g g^{-1} was reported in biologically treated hydrolysate derived from MC substrate. The previous study on co-culture of *C. acetobutylicum* with *Clostridium cellulolyticum* and *C. acetobutylicum* with *Clostridium thermocellum* produced yield of solvents 0.053 g g^{-1} and 0.3 g g^{-1} from substrate cellulose solka floc respectively [19, 46]. The productivities in the present study ranged from 0.28 to 0.53 g m^{-3} h^{-1} for thermochemically treated hydrolysates and 1.75 to 1.91 g m^{-3} h^{-1} for biologically treated hydrolysates. The results indicated that highest saccharide concentrations were released into hydrolysate by both thermochemical and biological approaches and also saccharides were used during fermentation **(Table 2)** but total solvents yield were very low. Several factors may cause cessation during fed-batch fermentation such as nutrient starvation, oxygen contamination in experimental bottles, toxicity of supplemented minerals, accumulation of undetermined fermentation products (such as acids) and culture degeneration due to toxicity [47]. It should be noted that there was no oxygen contamination throughout the experiment that were carried out in well-sealed glass bottles. Also, the large amounts of saccharides that were utilised during fermentation indicates *C. actobutylicum* flourished well on hydrolysates during fermentation and that the medium was devoid of oxygen contamination. There is another possibility that the culture apparently failed to switch from acidogenic to solventogenic, a phenomenon known as “acid crash”, which occasionally occurs in pH-uncontrolled batch fermentations [48] contributed to premature termination of fermentation due to

excess acid production. Therefore, further process optimisation is needed. To make the process (more) efficient, detoxification of hydrolysate and simultaneous product recovery will be the aim of our future study as suggested previously [13, 49].

The major issue with biological saccharification is slow saccharification depending on crystallinity of the substrates that often result in low yield of fermentable saccharides. The combination of a mild pre-treatment such as shockwave treatment with biological saccharification could potentially increase saccharification, thereby, improve fermentation. Therefore, our future study will be focused on the combination of shockwave pre-treatment and biological saccharification as suggested by Marausek *et al* [50].

4. Conclusions

In this study, for the first time, it was successfully demonstrated that both thermochemical and biological pre-treatments approaches produced fermentable saccharides and subsequently fermented to biofuels (ethanol, butanol and H_2) using *C. acetobutylicum*. This study also demonstrated the great potential of *C. acetobutylicum* as a future biofuel-generating candidate from lignocellulosic feedstock since it can utilise a wide variety of sugars in fermentation.

In first approach, thermochemical saccharification with 100 mol m^{-3} H_2SO_4 provided high degree of saccharification, thus higher subsequent biofuels and H_2 production were reported but overall solvents yield were lower (0.0069 g g^{-1}) compared to H_2O (0.045 g g^{-1}) and 200 mol m^{-3} NaOH (0.01 g g^{-1}). The result indicates that although highest saccharides released into the hydrolysates during 100 mol m^{-3} H_2SO_4 treatment and utilised during fermentation, the overall conversion to solvents were

very low **(Table 2)**. Therefore, detoxification of hydrolysate prior to fermentation and simultaneous product recovery is required to achieve high degree of fermentation. Similarly, in a second approach, biological saccharification and fermentation with *F. succinogenes* and *C. acetobutylicum* were successfully achieved and produced solvents but the total solvents yield was lower. The highest solvents yield were obtained in biological MC hydrolysates (0.103 g g^{-1}) compared to ASC (0.07 g g^{-1}) and MG (0.09 g g^{-1}). Therefore, the results of this study confirm our hypothesis that biological saccharification is just as promising as thermochemical saccharification strategies for lignocellulosic biofuel production. Although, the two anaerobic bacteria used in this study are promising candidates for a future CBP development by sequential co-culture fermentation of lignocellulosic wastes, the further optimisation of this technique is required. This would then also require deep subsequent financial appraisal.

With the present knowledge, two areas that needs to be focused on in order to achieve a viable biofuel production process. Firstly, thermochemical pre-treatment requires development of robust fermentation step (i.e. requires industrially robust fermentation microorganisms) due to the presence of inhibitors. Secondly, biological saccharification requires a combination of mild pre-treatment such as shockwave pre-treatment in order to improve saccharification and fermentation. Future work will be focused on a biological saccharification approach since biological saccharification and fermentation can provide a potentially eco-friendly technology for lignocellulosic biofuel generation.

Acknowledgments

The authors wish to acknowledge the Ministry of Social Justice and Empowerment Govt. of India for providing financial support under National Overseas Fellowship (Grant No. 11015/22/2008-SCD-V). The EPSRC (EP/E036252/1, EP/E053556/1) and SUNLIBB (SUNLIBB—Sustainable Liquid Biofuels from Biomass Biorefining, European Union Project 251132, FP7-ENERGY-2009-BRAZIL) programme are also acknowledged. We are grateful to Prof. Paul Weimer of the US Dairy Forage Research Centre (USDA – Agricultural Research Service - ARS; Wisconsin, USA) for providing *F. succinogenes* S85 (ATCC 19169). The authors wish to thank Dr. Narciso Couto and Dr. Esther Karunakaran for critically reading the manuscript.

The following are the supplementary data related to this article:

Appendix A. Supplementary data (DOCX)

Fig. S1. Growth profiles of *F. succinogenes* S85 (A) and *C. acetobutylicum* ATCC 824 (B) on different combinations of FS and CA media.

Fig. S2 Experimental set-up of the fermentation of thermochemically derived MG hydrolysate using *C. acetobutylicum*.

Fig. S3 Biological hydrolysis of lignocellulosic biomass hydrolysate and fermentation. A) Modified cellulose medium with MC as a substrate, B) Growth of *F. succinogenes* at 40 hrs of incubation (biofilm) and C) Fermentation (*F. succinogenes* plus *C. acetobutylicum*) at 120 hrs.

Appendix B. Supplementary data (XLSX)

Raw data for changes in concentration of saccharides and metabolites during fermentation.

References

[1] I. Egüés, C. Sanchez, I. Mondragon, J. Labidi, Effect of alkaline and autohydrolysis processes on the purity of obtained hemicelluloses from corn stalks, Bioresour. Technol. 103(1) (2012) 239-248.

[2] M. Adsul, M. Singhvi, S. Gaikaiwari, D. Gokhale, Development of biocatalysts for production of commodity chemicals from lignocellulosic biomass, Bioresour. Technol. 102(6) (2011) 4304-4312.

[3] Y. Sun, J. Cheng, Hydrolysis of lignocellulosic materials for ethanol production: a review, Bioresour. Technol. 83(1) (2002) 1-11.

[4] J. Zhu, X. Pan, Woody biomass pretreatment for cellulosic ethanol production: technology and energy consumption evaluation, Bioresour. Technol. 101(13) (2010) 4992-5002.

[5] V. Chaturvedi, P. Verma, An overview of key pretreatment processes employed for bioconversion of lignocellulosic biomass into biofuels and value added products, 3 Biotech 3(5) (2013) 415-431.

[6] J. Maroušek, Biotechnological partition of the grass silage to streamline its complex energy utilization, Int. J. green energy. 11(9) (2014) 962-968.

[7] P. Alvira, E. Tomás-Pejó, M. Ballesteros, M. Negro, Pretreatment technologies for an efficient bioethanol production process based on enzymatic hydrolysis: a review, Bioresour. Technol. 101(13) (2010) 4851-4861.

[8] S.S. Hassan, G.A. Williams, A.K. Jaiswal, Emerging technologies for the pretreatment of lignocellulosic biomass, Bioresour. Technol. 262 (2018) 310-318.

[9] F. Sabathé, P. Soucaille, Characterization of the CipA scaffolding protein and in vivo production of a minicellulosome in *Clostridium acetobutylicum*, J. Bateriol. 185(3) (2003) 1092-1096.

[10] M.P. Raut, N. Couto, T.K. Pham, C. Evans, J. Noirel, P.C. Wright, Quantitative proteomic analysis of the influence of lignin on biofuel production by *Clostridium acetobutylicum* ATCC 824, Biotechnol. Biofuels 9(1) (2016) 113.

[11] L.D. Gómez, R. Vanholme, S. Bird, G. Goeminne, L.M. Trindade, I. Polikarpov, R. Simister, K. Morreel, W. Boerjan, S.J. McQueen-Mason, Side by side comparison of chemical compounds generated by aqueous pretreatments of maize stover, Miscanthus and sugarcane bagasse, BioEnergy Res. 7(4) (2014) 1466-1480.

[12] N. Qureshi, T.C. Ezeji, Butanol,'a superior biofuel'production from agricultural residues (renewable biomass): recent progress in technology, Biofuels Bioprod. Biorefin. 2(4) (2008) 319-330.

[13] H. Huang, V. Singh, N. Qureshi, Butanol production from food waste: a novel process for producing sustainable energy and reducing environmental pollution, Biotechnol. Biofuels 8(1) (2015) 147.

[14] E.M. Rubin, Genomics of cellulosic biofuels, Nature 454(7206) (2008) 841-845.

[15] E. Guedon, M. Desvaux, H. Petitdemange, Improvement of cellulolytic properties of *Clostridium cellulolyticum* by metabolic engineering, Appl. Environ. Microbiol. 68(1) (2002) 53-58.

[16] L.R. Lynd, P.J. Weimer, W.H. Van Zyl, I.S. Pretorius, Microbial cellulose utilization: fundamentals and biotechnology, Microbiol. Mol. Biol. Rev. 66(3) (2002) 506-577.

[17] A. Ranjan, V.S. Moholkar, Biobutanol: science, engineering, and economics, Int. J. Energy Res. 36(3) (2012) 277-323.

[18] F. Salimi, K. Zhuang, R. Mahadevan, Genome-scale metabolic modeling of a clostridial co-culture for consolidated bioprocessing, Biotechnol. J. 5(7) (2010) 726-738.

[19] E. Petitdemange, O. Fond, F. Caillet, H. Petitdemange, R. Gay, A novel one step process for cellulose fermentation using mesophilic cellulolytic and glycolytic Clostridia, Biotechnol. Lett. 5(2) (1983) 119-124.

[20] Y. Jiao, A. Navid, B.J. Stewart, J.B. McKinlay, M.P. Thelen, J. Pett-Ridge, Syntrophic metabolism of a co-culture containing *Clostridium cellulolyticum* and *Rhodopseudomonas palustris* for hydrogen production, Int J. Hydrogen Energ. 37(16) (2012) 11719-11726.
[21] A. Wang, N. Ren, Y. Shi, D.-J. Lee, Bioaugmented hydrogen production from microcrystalline cellulose using co-culture—*Clostridium acetobutylicum* X9 and *Ethanoigenens harbinense* B49, Int J. Hydrogen Energ. 33(2) (2008) 912-917.
[22] C. Béra-Maillet, Y. Ribot, E. Forano, Fiber-degrading systems of different strains of the genus Fibrobacter, Appl. Environ. Microbiol. 70(4) (2004) 2172-2179.
[23] M.P. Raut, E. Karunakaran, J. Mukherjee, C.A. Biggs, P.C. Wright, Influence of substrates on the surface characteristics and membrane proteome of *Fibrobacter succinogenes* S85, PloS one 10(10) (2015) e0141197.
[24] A.M. López-Contreras, A.A. Martens, N. Szijarto, H. Mooibroek, P.A. Claassen, J. van der Oost, W.M. de Vos, Production by *Clostridium acetobutylicum* ATCC 824 of CelG, a cellulosomal glycoside hydrolase belonging to family 9, Appl. Environ. Microbiol. 69(2) (2003) 869-877.
[25] E. Callaway, S. Martin, Effects of a *Saccharomyces cerevisiae* culture on ruminal bacteria that utilize lactate and digest cellulose, J. Dairy Sci. 80(9) (1997) 2035-2044.
[26] P.J. Weimer, Y. Shi, C.L. Odt, A segmented gas/liquid delivery system for continuous culture of microorganisms on insoluble substrates and its use for growth of *Ruminococcus flavefaciens* on cellulose, Appl. Microbiol. Biotechnol. 36(2) (1991) 178-183.
[27] H. George, J. Johnson, W. Moore, L. Holdeman, J. Chen, Acetone, isopropanol, and butanol production by *Clostridium beijerinckii* (syn. *Clostridium butylicum*) and *Clostridium aurantibutyricum*, Appl. Environ. Microbiol. 45(3) (1983) 1160-1163.
[28] R.S. Ramirez, M. Holtzapple, N. Piamonte, Fundamentals of biomass pretreatment at high pH, aqueous pretreatment of plant biomass for biological and chemical conversion to fuels and chemicals, Hoboken: Wiley (2013) 145-167.
[29] J.E. Wells, J.B. Russell, Y. Shi, P.J. Weimer, Cellodextrin efflux by the cellulolytic ruminal bacterium *Fibrobacter succinogenes* and its potential role in the growth of nonadherent bacteria, Appl. Environ. Microbiol. 61(5) (1995) 1757-1762.
[30] T.R. Zuroff, S.B. Xiques, W.R. Curtis, Consortia-mediated bioprocessing of cellulose to ethanol with a symbiotic *Clostridium phytofermentans*/yeast co-culture, Biotechnol. Biofuels 6(1) (2013) 59.
[31] L. Jones, J.L. Milne, D. Ashford, S.J. McQueen-Mason, Cell wall arabinan is essential for guard cell function, Proc. Natl. Acad. Sci. 100(20) (2003) 11783-11788.
[32] T.K. Pham, P.K. Chong, C.S. Gan, P.C. Wright, Proteomic analysis of *Saccharomyces cerevisiae* under high gravity fermentation conditions, J. Proteome Res. 5(12) (2006) 3411-3419.
[33] H. Bahl, W. Andersch, K. Braun, G. Gottschalk, Effect of pH and butyrate concentration on the production of acetone and butanol by *Clostridium acetobutylicum* grown in continuous culture, Eur. J. Appl. Microbiol. Biotechnol. 14(1) (1982) 17-20.
[34] F. Monot, J.-M. Engasser, H. Petitdemange, Influence of pH and undissociated butyric acid on the production of acetone and butanol in batch cultures of *Clostridium acetobutylicum*, Appl. Microbiol. Biotechnol. 19(6) (1984) 422-426.
[35] J. Gottschal, J. Morris, Non-production of acetone and butanol by *Clostridium acetobutylicum* during glucose-and ammonium-limitation in continuous culture, Biotechnol. Lett. 3(9) (1981) 525-530.
[36] K. Gao, L. Rehmann, ABE fermentation from enzymatic hydrolysate of NaOH-pretreated corncobs, Biomass Bioenerg. 66 (2014) 110-115.
[37] A.M. López-Contreras, P.A. Claassen, H. Mooibroek, W.M. De Vos, Utilisation of saccharides in extruded domestic organic waste by *Clostridium acetobutylicum* ATCC 824 for production of acetone, butanol and ethanol, Appl. Microbiol. Biotechnol. 54(2) (2000) 162-167.
[38] S. Wang, Y. Zhang, H. Dong, S. Mao, Y. Zhu, R. Wang, G. Luan, Y. Li, Formic acid triggers the "acid crash" of acetone-butanol-ethanol fermentation by *Clostridium acetobutylicum*, Appl. Environ. Microbiol. 77(5) (2011) 1674-1680.

[39] P. Kaparaju, M. Serrano, A.B. Thomsen, P. Kongjan, I. Angelidaki, Bioethanol, biohydrogen and biogas production from wheat straw in a biorefinery concept, Bioresour. Technol. 100(9) (2009) 2562-2568.
[40] B.C. Saha, L.B. Iten, M.A. Cotta, Y.V. Wu, Dilute acid pretreatment, enzymatic saccharification and fermentation of wheat straw to ethanol, Process Biochem. 40(12) (2005) 3693-3700.
[41] Q. He, H. Chen, Improved efficiency of butanol production by absorbent fermentation with a renewable carrier, Biotechnol. Biofuels 6(1) (2013) 1-9.
[42] M. Cui, J. Shen, Effects of acid and alkaline pretreatments on the biohydrogen production from grass by anaerobic dark fermentation, Int. J. Hydrogen Energ. 37(1) (2012) 1120-1124.
[43] H. Amiri, K. Karimi, Improvement of acetone, butanol, and ethanol production from woody biomass using organosolv pretreatment, Bioprocess Biosyst. Eng. 38(10) (2015) 1959-1972.
[44] M. Shao, H. Chen, Feasibility of acetone–butanol–ethanol (ABE) fermentation from Amorphophallus konjac waste by *Clostridium acetobutylicum* ATCC 824, Process Biochem. 50(8) (2015) 1301-1307.
[45] Y. Liu, P. Yu, X. Song, Y. Qu, Hydrogen production from cellulose by co-culture of *Clostridium thermocellum* JN4 and *Thermoanaerobacterium thermosaccharolyticum* GD17, Int. J. Hydrogen Energ. 33(12) (2008) 2927-2933.
[46] E. Yu, M.-H. Chan, J. Saddler, Butanol production from cellulosic substrates by sequential co-culture of *Clostridium thermocellum* and *C. acetobutylicum*, Biotechnol lett. 7(7) (1985) 509-514.
[47] T. Ezeji, N. Qureshi, H. Blaschek, Acetone butanol ethanol (ABE) production from concentrated substrate: reduction in substrate inhibition by fed-batch technique and product inhibition by gas stripping, Appl. Microbiol. Biotechnol. 63(6) (2004) 653-658.
[48] I. Maddox, E. Steiner, S. Hirsch, S. Wessner, N. Gutierrez, J. Gapes, K. Schuster, The cause of "acid crash" and "acidogenic fermentations" during the batch acetone-butanol-ethanol (ABE-) fermentation process. J. Mol. Microbiol. Biotechnol. 2(1) (2000) 95-100.
[49] N. Qureshi, V. Singh, S. Liu, T.C. Ezeji, B. Saha, M. Cotta, Process integration for simultaneous saccharification, fermentation, and recovery (SSFR): production of butanol from corn stover using *Clostridium beijerinckii* P260, Bioresour. Technol. 154 (2014) 222-228.
[50] J. Maroušek, S. Itoh, O. Higa, Y. Kondo, M. Ueno, R. Suwa, J. Tominaga, Y. Kawamitsu, Enzymatic hydrolysis enhanced by pressure shockwaves opening new possibilities in Jatropha Curcas L. processing, J. Chem. Technol. Biotechnol. 88(9) (2013) 1650-1653.

Figure captions

Fig. 1. Overview of experimental design. MG; *Miscanthus giganteus,* ASC; acid swollen cellulose, MC; microcrystalline cellulose.

Fig. 2. Alcoholic fermentation of thermochemically derived MG hydrolysates (treatments; H_2O, 100 mol m^{-3} H_2SO_4 and 200 mol m^{-3} NaOH) by *C. acetobutylicum*. A) ethanol, B) butanol and C) H_2 gas. Samples were taken at 80

hours (▒) and 120 hours (■) of fermentation. Data were taken from biological triplicates. Error bars indicate the standard error of the mean.

Fig. 3. Syntrophic growth of *F. succinogenes* and *C. acetobutylicum* on modified media. Rod shaped cells represent *C. acetobutylicum* and coccoidal shaped cells represent *F. succinogenes*.

Fig. 4. Alcoholic fermentation of biologically derived lignocellulosic biomass hydrolysate by *C. acetobutylicum*. A) ethanol, B) butanol and C) H_2 gas. Samples were taken at 80 hours (▒) and 120 hours (■) of fermentation. Data were taken from biological triplicates. Error bars indicate the standard error of the mean.

Fig. 1

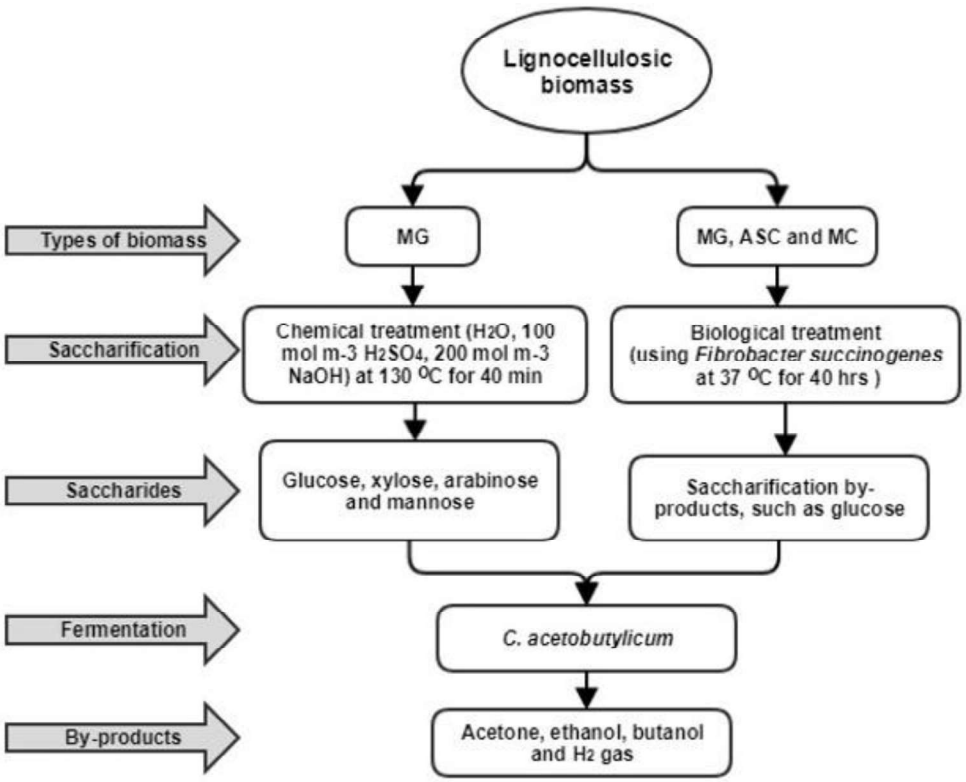


Fig. 2

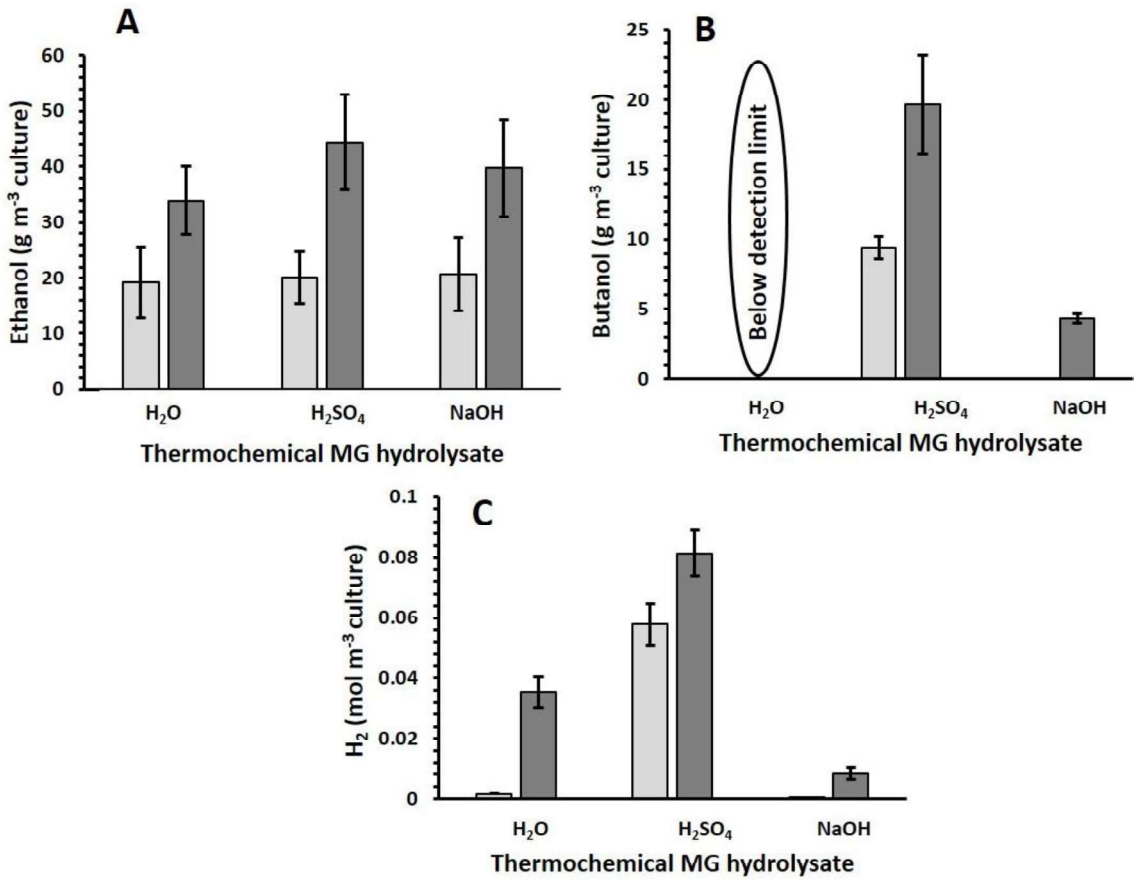

A
Ethanol (g m-3 culture)
60
50
40
30
20
10
0
H2O
H2SO4
NaOH
Thermochemical MG hydrolysate
B
Butanol (g m-3 culture)
25
20
15
10
5
0
Below detection limit
H2O
H2SO4
NaOH
Thermochemical MG hydrolysate
C
H2 (mol m-3 culture)
0.1
0.08
0.06
0.04
0.02
0
H2O
H2SO4
NaOH
Thermochemical MG hydrolysate

Fig. 3

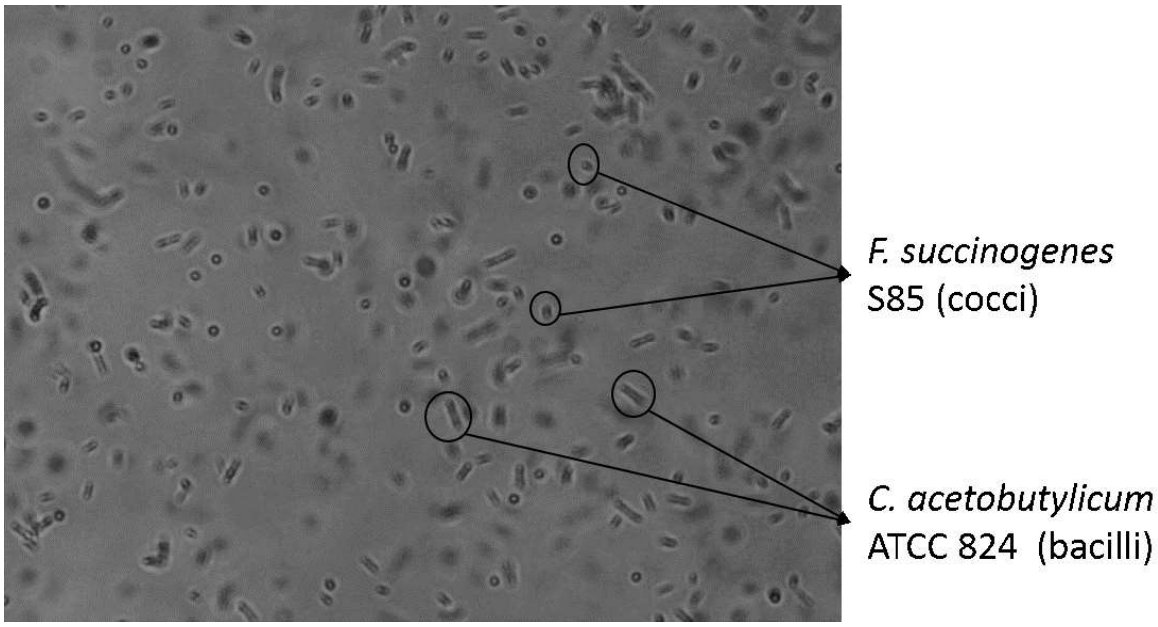
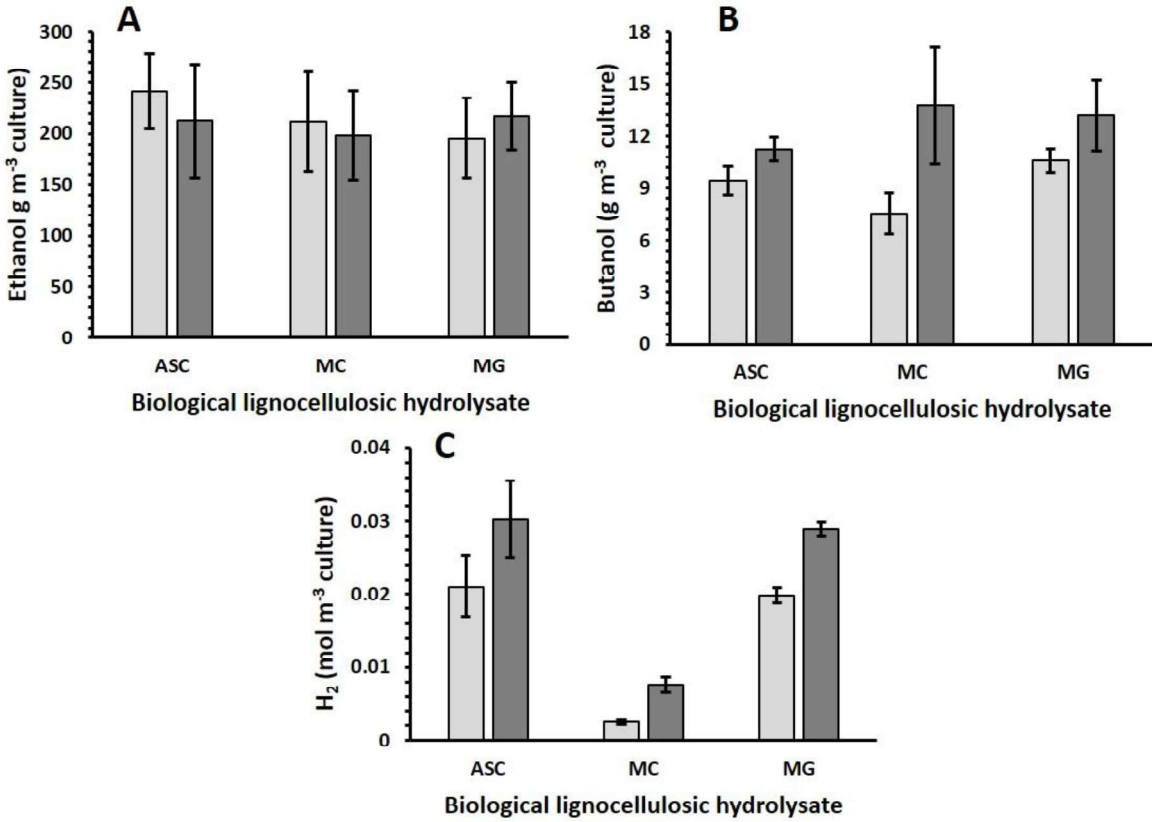

F. succinogenes
S85 (cocci)
C. acetobutylicum
ATCC 824 (bacilli)

Fig. 4


A
Ethanol g m-3 culture)
300
250
200
150
100
50
0
ASC
MC
MG
Biological lignocellulosic hydrolysate
B
Butanol (g m-3 culture)
18
15
12
9
6
3
0
ASC
MC
MG
Biological lignocellulosic hydrolysate
C
H2 (mol m-3 culture)
0.04
0.03
0.02
0.01
0
ASC
MC
MG
Biological lignocellulosic hydrolysate

767

768

Table 1 Summary of relationship between concentration of saccharides and fermentation by-products formation

Saccharides/ metabolites	Saccharides concentration (initial; 0 hrs, and final; 120 hrs fermentation)								Fermentation products					
	Glucose (g m^{-3})		Xylose (g m^{-3})		Arabinose (g m^{-3})		Mannose (g m^{-3})		Ethanol (g m^{-3})		Butanol (g m^{-3})		H_2 (mol m^{-3})	
Treatments	**Initial**	**Final**	**Initial**	**Final**	**Initial**	**Final**	**Initial**	**Final**	**80 hrs**	**120 hrs**	**80 hrs**	**120 hrs**	**80 hrs**	**120 hrs**
H_2O	155 ± 34.69	2 ± 0.47	170 ± 30	0	114 ± 23	6 ± 0.48	311 ± 43	0	19.2 ± 6.4	33.9 ± 6	0	0	0.0018 ± 8.4 E-5	0.0353 ±005
100 mol m^{-3} H_2SO_4	607 ± 106	1 ± 0.09	6229 ± 699	28 ± 0.29	1627 ± 249	13 ± 1.2	1399 ± 169	248 ± 6.2	20.1 ± 4.6	44.4 ± 8.5	9.4 ± 0.8	19.7 ± 3.5	0.058 ± 0.006	0.0815 ± 007
200 mol m^{-3} NaOH	365 ± 56	1 ± 0.29	2199 ± 226	2 ± 0.71	980 ± 140	3 ± 3.8	551 ± 117	35 ± 14	20.6 ± 6.5	39.7 ± 8.6	0	4.3 ±0.33	0.0005 ± 1.6 E-5	0.0084 ± 002

Table 2 Comparison of total solvents (ABE or BE) yield and productivity among thermochemically and biologically pre-treated hydrolysate

Thermochemical pre-treatment of MG	**Total saccharides used (g m^{-3})**	**Total solvents produced (g m^{-3})**	**Total yield of solvents (g g^{-1})**	**Productivity of solvents (g m^{-3} h^{-1})**
H_2O	742	33.9	0.045	0.28
100 mol m^{-3} H_2SO_4	9572	64.1	0.0069	0.53
200 mol m^{-3} NaOH	4054	44	0.01	0.36
Biological pre-treatment of lignocellulosic substrate				
ASC	3349	223	0.066	1.85
MC	2040	211.7	0.103	1.75
MG	2504	230.2	0.091	1.91

Appendix A. Supplementary data (DOCX)

Alcoholic fermentation of thermochemical and biological hydrolysates derived from *Miscanthus* biomass by *Clostridium acetobutylicum* ATCC 824

Mahendra P. Raut[a], Trong K. Pham[a], Leonardo D. Gomez[b], Ioanna Dimitriou[c] and Phillip C. Wright[d] *

Author affiliation:

[a] Department of Chemical and Biological Engineering, The University of Sheffield, Mappin Street, Sheffield, S1 3JD, UK

[b] CNAP, Biology Department, University of York, Heslington, York, YO10 5DD, UK

[c] Department of Chemical and Environmental Engineering, The University of Nottingham, University Park, Nottingham, NG7 2RD, UK

[d] School of Chemical Engineering and Advanced Materials, c/- Faculty of Science, Agriculture and Engineering, Devonshire Building, Newcastle University Newcastle upon Tyne, NE1 7RU, UK

*Corresponding author information: E-mail: phillip.wright@newcastle.ac.uk Ph: +44 (191) 208 4960

Number of pages: 4

Number of figures: 3

Medium optimisation for co-culture development

To obtain the modified media, we prepared 6 media bottles of each FS and CA media with 5 g L^{-1} glucose as a carbon source as discussed in section 2.2 & 2.3.1. Then, we combined both the media (FS to CA (v/v)) to obtain the ratio of 100 % FS, 20 % FS plus 80 % CA, 40 % FS plus 60 % CA, 60 % FS plus 40 % CA, 80 % FS plus 20 % CA and 100 % CA. There were two sets of these combinations prepared. All the combinations were prepared in an anaerobic chamber in pre-sterilized 125 mL serum bottles caped with butyl rubber and crimp sealed. These modified media were then inoculated with *F. succinogenes (*OD_{675} =0.72) and *C. acetobutylicum* (OD_{600} = 1.2), and grown on their respective media with glucose as a carbon source. The growth of both bacteria was monitored in their respective sets of media by measuring OD at 675_{nm} for *F. succinogenes* and at 600_{nm} for *C. acetobutylicum*. From the reading obtained from both bacteria, the combination of 40 % FS plus 60 % CA media was considered as a modified media for the growth of both bacteria.

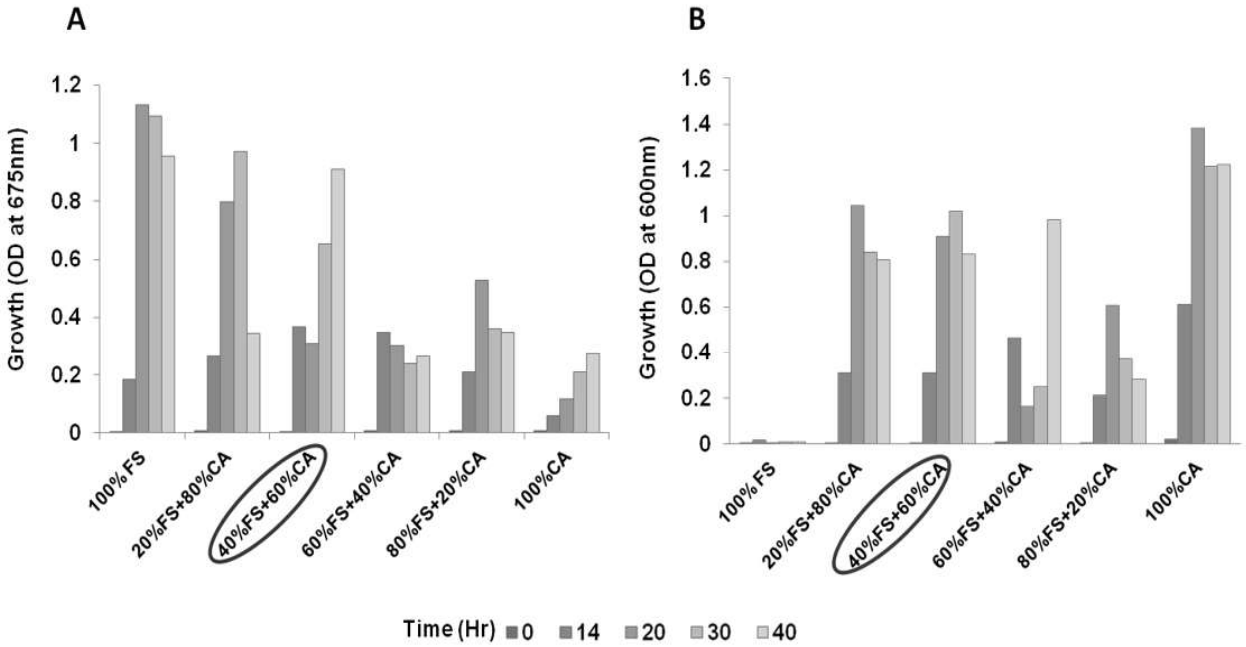


Fig. S 1. Growth profiles of *F. succinogenes* S85 (A) and *C. acetobutylicum* ATCC 824 (B) on different combinations of FS and CA media.

Fig. S2 Experimental set-up of the fermentation of miscanthus biomass hydrolysate using *C. acetobutylicum.*

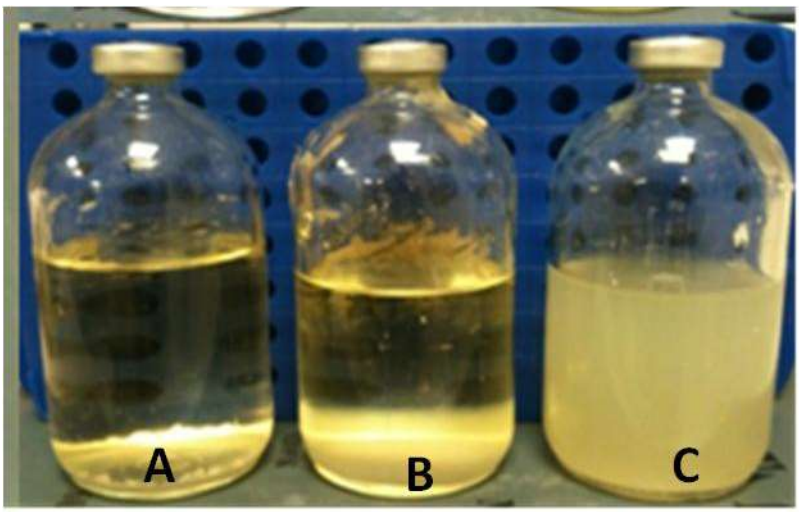


Fig. S3 Biological hydrolysis of cellulose and fermentation. A) Modified cellulose medium with MC cellulose as substrate, B) Growth of *F. succinogenes* at 40hrs of incubation (biofilm) and C) Fermentation (*F. succinogenes* plus *C. acetobutylicum*) at 120 hrs.

Appendix B. Supplementary data (XLSX)

Substrate sugars concetration

Average values derived from 3 biological replicates, SD means standard deviation.

Glucose (g/m^3) culture	Before fermentation		After fermentation	
	Average	SD	Average	SD
H_2O	154.84	34.69	1.72	0.47
100 mol m^3 H_2SO_4	607.44	106.79	1.07	0.10
200 mol m^3 NaOH	364.74	56.74	0.56	0.30

Xylose (g/m^3) culture	Before fermentation		er fermentar fermentation	
	Average	SD	Average	SD
H_2O	169.66	30.36	0.00	0.00
100 mol m^3 H_2SO_4	6229.40	699.18	27.72	0.29
200 mol m^3 NaOH	2199.12	226.58	2.01	0.72

Arabinose (g/m^3) culture	Before fermentation		After fermentation	
	Average	SD	Average	SD
H_2O	113.55	23.84	5.78	0.49
100 mol m^3 H_2SO_4	1626.92	249.43	12.71	1.22
200 mol m^3 NaOH	980.15	140.85	3.42	3.88

Mannose (g/m^3) culture	Before fermentation		er fermentar fermentation	
	Average	SD	Average	SD
H_2O	311.18	42.90	0.00	0.00
100 mol m^3 H_2SO_4	1399.47	169.71	248.35	6.23
200 mol m^3 NaOH	551.44	117.26	35.25	14.89

Cellulose substrate (g/m^3) culture	Before fermentation		After fermentation	
	Average	SD	Average	SD
ASC	5123.00	110.15	1774.67	351.97
MC	5132.00	190.09	3092.00	433.42
MG	5085.00	57.74	2584.67	774.40