Evaluation of different lignocellulosic biomass pretreatments by phenotypic microarray-based metabolic analysis of fermenting yeast

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HIGHLIGHTS
- Efficacy of pre-treatments on different lignocellulosic materials tested.
- Phenotypic microarray was used to access fermentation.
- Alkaline system liberated more sugar but hydrolysates not as fermentable.
- Acid system had best fermentability.
- Acetic acid and furfural present reduced ethanol production to 70% theoretical yield.

GRAPHICAL ABSTRACT

Advanced generation biofuel production from lignocellulosic material (LCM) was investigated. A range of different thermo-chemical pre-treatments were evaluated with different LCM. The pre-treatments included; alkaline (5% NaOH at 50°C), acid (1% H₂SO₄ at 121°C) and autohydrolytical methods (200°C aqueous based hydrothermal) and were evaluated using samples of miscanthus, wheat-straw and willow. The liberation of sugars, presence of inhibitory compounds, and the degree of enhancement of enzymatic saccharification was assessed. The suitability of the pre-treatment generated hydrolysates (as bioethanol feedstocks for Saccharomyces cerevisiae) was also accessed using a phenotypic microarray that measured yeast metabolic output. The use of the alkaline pre-treatment liberated more glucose and arabinose into both the pre-treatment generated hydrolysate and also the hydrolysate produced after enzymatic hydrolysis (when compared with other pre-treatments). However, hydrolysates derived from use of alkaline pre-treatments were shown to be unsuitable as a fermentation medium due to issues with colloidal stability (high viscosity). Use of acid or autohydrolytical pre-treatments liberated high concentrations of monosaccharides regardless of the LCM used and the hydrolysates had good fermentation performance with measurable yeast metabolic output. Acid pre-treated wheat straw hydrolysates were then used as a model system for larger scale fermentations to confirm both the results of the phenotypic microarray and its validity as an effective high-throughput screening tool.

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1. Introduction

Current environmental, economic, and social concerns regarding the sustainability of use of fossil fuels have led to considerable research into alternative energy resources such as liquid biofuel production from various biomass types (Balat, 2011; Chundawat et al., 2011). Whilst first generation biofuel production from energy crops such as sugar cane and corn have had some success, concerns over use of potential food sources for the production of transportation fuels has been highlighted (Rathmann et al., 2010). Second generation biofuel production has been developed to minimise this issue through the use of lignocellulosic biomass (utilising the structural polysaccharide components found within the cell wall material), as this material cannot be directly used for human food production (Carvalheiro et al., 2011a).

However, there are considerable technical difficulties to be overcome in order to utilise lignocellulosic feedstocks due to their recalcitrant nature which resists biotic degradation (such as via enzymatic hydrolysis routes). As a consequence of this recalcitrance, the production of second generation biofuels from lignocellulosic biomass (such as wheat straw, willow, and miscanthus) normally requires chemical or thermal pre-treatment prior to enzymatic saccharification in order to boost fermentable sugar yields (Binod et al., 2012; Galbe, 2012). The aim of the pre-treatment is to improve enzymatic access to the cellulosic component within the lignocellulosic matrix through solubilisation or fractionation of various components. Different pre-treatments target different components with autohydrolytical (entirely aqueous based hydrothermal techniques) and acid catalysed hydrothermal pre-treatments primarily targeting hemicellulose removal whilst alkaline (caustic) reagents primarily targets lignin removal (Girio et al., 2010; Banerjee et al., 2011). Pre-treatment has been highlighted as the most energy intensive stage of the second generation biofuel process, and optimising protocols in terms of minimising chemical and energy inputs is crucial for any potential large scale production (Yang and Wyman, 2008).

In addition to energy efficiency, the use of excessive pre-treatment reaction conditions can result in the formation of compounds which act as inhibitors to downstream processes including enzymatic saccharification and fermentations (Palmqvist et al., 1998; Chheda et al., 2007; Allen et al., 2010). These compounds are often the result of thermal or chemical degradation of liberated sugars (to furan based inhibitors) or lignin (to phenolic/hydroxycinnamic acid based inhibitors). Hydrolysates are generated directly from the pre-treatment step (the liquid fractions) and they can contain significant concentrations of these compounds. This can present significant problems (such as long yeast lag phases, poor attenuation, and sub-optimal ethanol yields) for any fermentations conducted using these hydrolysates. These hydrolysates can contain a significant quantity of supplementary fermentable sugars (in addition to those liberated after enzymatic hydrolysis routes). As such, their use is crucial for maximising the use of lignocellulosic material (LCM) as a biofuel substrate. Consequently, the assessment of the fermentation performance of these hydrolysates is a key factor in identifying issues which may reduce the efficiency of any proposed biofuel production systems using LCM. Additionally, identifying pre-treatment systems that generate excessive quantities of inhibitors is a key factor in the formulation of effective advanced generation biofuel production processes.

Wheat-straw (Triticum aestivum L.) is a by-product from wheat production and was chosen due to its status as the largest biomass feedstock in the Europe (Saha and Cotta, 2006). Miscanthus × giganteus or miscanthus is an Asian perennial rhizomatous grass and is potentially a dedicated energy crop (Bauen et al., 2010). This was chosen due to its current major use as a fuel for heat generation in power stations (DEFFRA, 2007) and also as a representative of herbaceous, perennial biomass (McKendry, 2002). Perennials are often considered superior to annuals in terms of their lower pesticide and fertiliser requirements and their superior usage of nutrients (Jørgensen, 2011). Short rotation coppice (SRC) willow was also chosen as another candidate due to its high growth yields and again low fertiliser requirements as with miscanthus (Ray, 2012) and as a model for the woody biomass type (Sticklen, 2008).

Three different pre-treatments were selected and applied on commonly available LCMs which have all previously been highlighted as potential energy crops in the UK (Ghithero, 2013a; Ghithero et al., 2013b). The pre-treatments were all chosen as effective for LCM and consisted of an acid hydrothermal system; 1% HSO₄ at 121°C (Wilkinson et al., 2014a), an alkaline system; 5% NaOH at 50°C (Wilkinson et al., 2014b), and finally an autohydrolytical system; 200°C aqueous based (Wilkinson et al., 2015). Use of sodium hydroxide as an alkali pre-treatment has been well established and successful, as alkali does not cause sugar degradation (Chang and Holtzapple, 2000). Dilute acid pre-treatments reduce hemicellulose to its monomeric sugars making cellulose more accessible (Nguyen et al., 2000) and use of autohydrolytical methods causes hemicellulose to become solubilised making the cellulose more accessible (Chandra et al., 2007).

This paper evaluated the efficacy of different pre-treatment protocols on various LCMs. Efficacy was determined in terms of differences in liberated sugar yield (both directly into the pre-treatment generated hydrolysate and also post cellulosolytic enzymatic saccharification), the degree of formation of metabolically inhibitory compounds, and the subsequent fermentation performance of the pre-treatment generated hydrolysates. The fermentation performance was then assessed using a phenotypic microarray (PM) as a novel, rapid screening tool that has previously been used to measure yeast metabolic output (Greetham, 2014; Wimalasena et al., 2014). To the author’s knowledge, very few studies have attempted to high-throughput screening the fermentability of different biofuel feedstocks. The PM results were then confirmed using large scale fermentations using wheat straw (with acid pre-treatment) as a model system. This validated the use of the PM as a novel, high-throughput screening tool for identifying issues with fermentability of biofuel feedstocks.

2. Materials and methods

2.1. Yeast strain and growth conditions

Saccharomyces cerevisiae NCYC 2592 (wwwNCYC.co.uk) was maintained on agar containing 10 g/L yeast extract, 20 g/L peptone, 20 g/L glucose, and 20 g/L agar (YPD agar) and grown in 10 g/L yeast extract, 20 g/L peptone, and 20 g/L glucose (YPD) in an orbital shaker (180 rpm) at 30°C under aerobic conditions.

2.2. Raw materials and inhibitors

Inhibitory chemicals such as acetic, formic, p-coumaric, and ferulic acids, furfural, 5-hydroxymethylfurfural (HMF), syringaldehyde, and vanillin were all supplied by Sigma (Dorset, UK). Other chemicals were standard laboratory reagents. Wheat straw was harvested at the University of Nottingham. Willow and miscanthus were harvested at Rothamsted (BBSRC funded) and details of the harvest have been published previously (Ray, 2012). Willow and miscanthus were harvested as part of field trials conducted under the BBSRC’s BBSC renewal initiative with site permission from the Lawes Trust; grid coordinates 51.8186’N and 0.3798’W.

2.3. Pre-treatments of LCM (wheat straw, miscanthus, and willow)

Acid catalysed hydrothermal pre-treatments were conducted using the protocol described previously (Wilkinson et al., 2014a). Biomass (50 g dry weight) was added to 500 mL 1% H₂SO₄ (w/v) in a screw-capped glass bottle (1L) to give the required solids-loading (10% w/v). This was then heated at 121°C for 30 min using a 40L bench top autoclave (Piorclave, Tactrol 2, RSC/E, UK). Alkaline pre-treatments were conducted as described previously (Wilkinson et al., 2014b). 500 mL borosilicate glass bottles with the appropriate amount of biomass (25 g) and caustic reagent (5% NaOH w/w) required to achieve the 10% (w/v) solids loading were incubated at 50°C for 12 h in a GD100 water bath (Grant, UK). Microwave-assisted autohydrolytical pre-treatment was conducted using the described protocol (Wilkinson et al., 2015c). A Monowave 300 microwave synthesis reactor (Anton Paar GmbH, Gratz, Austria) was used. Glass G30 (30 mL) microwave reaction vessels (Anton Paar GmbH, Gratz, Austria) with the appropriate amount of biomass (2.0 g) and water (20 mL) required to achieve the 10% (w/v) solids loading were heated at 200°C for 5 min.

After pre-treatment, samples were centrifuged at 3500 × g for 10 min and the supernatant was removed for analysis of sugars and known
inhibitory compounds. The residual biomass was then re-suspended in reverse osmosis (RO) water (20 mL). The three different re-suspended pre-treatment samples all had widely different pH values: pH 14 (± 0.3) for the alkaline sample, pH 2 (± 0.3) for the acid sample, and pH 4 (± 0.3) for the autohydrolytical sample. As such, all were adjusted to pH 5.0 (± 0.1) with either glacial acetic acid or 40% NaOH (w/v) and then exhaustively washed with RO water (by repeated re-suspension and centrifugation at 3500 × g for 10 min, discarding the supernatant each time). The remaining residues were then oven-dried overnight at 60°C prior to enzymatic saccharification. All experiments were conducted in triplicate.

2.4. Enzymatic saccharification of pre-treated residues

All enzyme digestions of pre-treatment residues were conducted using 24 h incubation periods at 50°C with agitation at 150 rpm (MaxQ 4358 shaking incubator, Thermo Scientific, UK). The assessment of the efficacy of different pre-treatment processes was conducted using a low solids-loading protocol (0.5% w/v) with lyophilised Celluclast® cellulase from Trichoderma reesei (ATCC 26921, Sigma-Aldrich, UK) using an excess of enzyme (50 FPU/g biomass) to determine the maximum sugar concentration obtainable. Pre-treated residue (200 mg) was mixed with 40 mL of a 1 g/L Celluclast® solution in 50 mM sodium citrate buffer (adjusted to pH 4.8 via glacial acetic acid) and incubated at 50°C for 24 h. Following the incubation period, the samples were centrifuged at 1500 × g for 10 min and the supernatant was sampled for quantification of sugars via ion chromatography (IC). The FPU was determined according to the method described by Ghose (1987). All experiments were conducted in triplicate.

2.5. Quantification of total monosaccharide content of pre-treated residues

Total glucose, xylose, and arabinose concentrations were quantified from total sugar analysis using IC after complete acid hydrolysis of the pre-treated residues using the protocol described previously (Wilkinson et al., 2014b). Dried biomass (30 mg) was weighed into a heat resistant Pyrex reaction vessel (50 mL). To each tube was added 1 mL of 12 M H2SO4 and the contents were incubated at 37°C in a water bath for 1 h. Water (11 mL) was added to dilute the acid concentration to 1 M and the contents were further incubated at 100°C for 2 h. The resulting solutions were then syringe-filtered (GF/C 25 mm filter diameter/1.2 μm pore size, Whatman, USA) and the concentration of monosaccharides was quantified by IC as described in the Section 2.8.

2.6. High performance chromatography (HPLC) and IC

2.6.1. Quantification of weak acid based inhibitors present in the pre-treatment generated hydrolysates

HPLC (utilising an AS-2055 Intelligent Auto-sampler and a PU-1580 Intelligent HPLC Pump; Jasco, Japan) was used for the analysis of acetic and formic acid. An aliquot (20 μL) of the hydrolysate (the liquid fraction generated directly from the pre-treatment step) was injected onto a 250 x 4.6 mm Synergi Hydro-RP column (Phenomenex, Macclesfield UK). The compounds were eluted with 20 mM potassium dihydrogen phosphate buffer (pH 2.5) at a flow rate of 1 mL/min and detected at 220 nm using a Spectro Monitor 3000 UV spectrophotometer (Milton Roy, Stone, UK). The amounts of acetic and formic acid were determined by peak area comparison (Azur software, Jasco, Great Dunmow UK) with authentic standards.

2.6.2. Quantification of furan and phenolic based inhibitors present in the pre-treatment generated hydrolysates

Furan and phenolic based inhibitors were quantified using HPLC with UV detection at 280 nm using the protocol described previously (Wilkinson et al., 2014b).

2.6.3. Quantification of monosaccharides in the pre-treatment generated hydrolysates and the feedstocks produced after enzymatic saccharification of pre-treated LCM

Liberated sugars were quantified via IC using an ICS 3000 system (Dionex, USA) fitted with a CarboPac PA20 column (150 mm x 3.0 mm; Dionex, USA) with pulsed amperometric electrochemical detection (PAD) using the method described by Wilkinson et al. (2014b).

2.7. Phenotypic microarray (PM) analysis

The Biolog OmniLog (Biolog, Hayward, CA, USA) was used as a rapid screening tool to measure the metabolic output of the yeast when cultured in the pre-treatment generated hydrolysates. This was primarily to evaluate the response of the yeast to the inhibitors present in the hydrolysates and to identify any issues with fermentation performance. The fermentation performance of the feedstocks generated after enzymatic saccharification was not evaluated. Instead, this study concentrated specifically on the pre-treatment generated hydrolysate. As the biomass had been exhaustively washed after pre-treatment (before the enzymatic saccharification step) the feedstocks subsequently produced would have contained negligible concentrations of inhibitors.

Biolog growth medium was prepared using 0.67% (w/v) yeast nitrogen base (YNB) supplemented with 6% (w/v) glucose, and 0.2 μL of tetrazolium redox dye (dye D; specific for fungi) (Biolog, Hayward, CA, USA). Final volume was made up to 30 μL using RO sterile distilled water and aliquoted to individual wells. Microarray analysis experiments on the effects of acetic acid and furfural were set up as above but the amount of RO sterile distilled water added was modified to account for the presence of the inhibitors. Stock solution (1 M) of acetic acid, was prepared using RO water; furfural was prepared as 1 M stock solutions in 100 % ethanol. Hydrolysates were spiked with the appropriate concentrations of glucose to give a 6% final solution and 0.2 μL of dye D was added. Strains were prepared for inoculation into the PM assay plates as follows. Glycerol stocks stored at -80°C were streaked onto YPD plates and incubated at 30°C for approximately 48 h. Two to three colonies from each strain were re-streaked to one well of a 96-well plate containing 500 μL of YPD and incubated overnight at 30°C. Cells were then inoculated into sterile water in 20 × 100 mm test tubes and adjusted to a transmittance of 62% (%1×10⁶ cells/mL) using a Biolog turbidimeter (Biolog, USA). Cell suspensions for the inoculums were prepared by mixing 125 μL of the above cells with IFY buffer™ (Biolog, USA) and the final volume was adjusted to 3 mL using RO sterile distilled water. Next 90 μL of the above mix was inoculated into each well in a Biolog 96-well plate. Anaerobic conditions were created using Oxygen absorbing packs (Mitsubishi AnaeroPak™, Pack-Aanaero, Mitsubishi Gas Chemicals, Tokyo, Japan) with an anaerobic indicator (Oxoid, Basingstoke, UK) and the plates were created using Oxygen absorbing packs (Mitsubishi AnaeroPak™, Pack-Aanaero, Mitsubishi Gas Chemicals, Tokyo, Japan) with an anaerobic indicator (Oxoid, Basingstoke, UK) and the plates were placed inside PM gas bags (Biolog, USA). The plates were then placed in the OmniLog reader and incubated for 50 h at 30°C. The OmniLog reader photographed the PM plates at 15 min intervals, and converted the pixel density in each well to a signal value using cell growth and dye conversion. Dye reduction which reflects metabolic activity of cells was defined here as the redox signal intensity. After completion of the run, the signal data was compiled and exported from the Biolog software using Microsoft® Excel. In all cases, a minimum of three replicate PM assay runs were conducted, and the mean signal values are presented. All experiments were conducted in triplicate.

2.8. Confirmation of phenotypic microarray results using larger scale fermentations

Fermentations using pre-treatment generated hydrolysates were conducted in 180 mL fermentation vessels (FVs). Cryopreserved yeast colonies were streaked onto YPD plates and incubated at 30°C for 48 h. These were then transferred to 200 mL of YPD and grown for 48 h in a 500 mL conical flask shaking at 30°C. Cells were harvested and washed three times with sterile RO water and then re-suspended in 5 mL of sterile water. Under control conditions, 1.5 × 10⁷ cells/mL were inoculated in...
92.5 mL of medium containing 4% glucose, 2% peptone, 1% yeast extract with 7.5 mL RO water. 92.5 mL of hydrolysate was spiked with 7.5 mL of an 80% glucose stock to give a final glucose concentration of 6% and buffered to a starting pH of 5 using 2M NaOH. Anaerobic conditions were prepared using a sealed butyl plug (Fisher, Loughborough, UK) and aluminium caps (Fisher Scientific). A hypodermic needle attached with a Bunsen valve was purged through rubber septum to facilitate the release of CO₂. All experiments were performed in triplicate and weight loss was measured at each time point. Fermentations were conducted at 30°C, with orbital shaking at 200 rpm. All experiments were conducted in triplicate.

3. Results and discussion

Three different pre-treatments were employed on three commonly available LCMs. The LCMs chosen have all previously been highlighted as potential energy crops in the UK (Glithero, 2013a; Glithero et al., 2013b) and this study looked at the efficacy of the pre-treatments in terms of sugars liberated, presence of inhibitory compounds, pH, and yeast fermentation performance.

3.1. Liberation of sugars from LCM using a range of pre-treatments.

The assessment of liberation of monomeric sugars from LCM into the hydrolysate (sugars liberated directly into the liquid fraction generated from the pre-treatment) was conducted following three pre-treatments. Hydrothermal pre-treatment (employing 1% H₂SO₄ at 121°C for 30 min) liberated significantly higher concentrations of xylose, arabinose, and glucose when compared with use of alkaline (5% NaOH at 50°C) or hydrothermal (200°C microwave-assisted) pre-treatment methods regardless of the LCM used (Fig. 1A).

Fig. 1 Liberation of sugars (directly into the liquid fraction generated from pre-treatment) from Miscanthus, wheat and willow using different pre-treatments and different lignocellulosic materials: (A) Liberation of monomeric sugars (xylose, arabinose, and glucose) by NaOH, H₂SO₄ and hydrothermal (pre-treatment fraction after NaOH, H₂SO₄, and hydrothermal pre-treatment methods (200°C) on wheat, willow, or Miscanthus. Data are represented as % theoretical sugar yields post enzymatic hydrolysis. Data are representative of triplicate values with standard deviation shown.

3.2. Sugar yields from pre-treated biomass after enzymatic saccharification

Assessment of the sugar levels liberated after enzymatic saccharification from the various pre-treated residues indicated that similar theoretical glucose yields (ca. 65%) were achieved from all three biomass types using either acid (1% H₂SO₄ at 121°C) or 200°C microwave-assisted hydrothermal pre-treatment protocols. However, use of the alkaline (5% NaOH at 50°C) pre-treatment liberated the highest glucose yield (ca. 75% theoretical) from all LCM biomass types (Fig. 1B). In addition, a significant arabinose concentration was detected only in pre-treatments using the alkaline system (which only equated to ca. 30% theoretical yield). Similar % theoretical xylose concentrations were observed from all biomass types using either the acid or hydrothermal pre-treatment systems. The alkaline pre-treatment was the only pre-treatment to exhibit specific LCM biomass type related variability in the % theoretical xylose concentrations achieved, with willow and Miscanthus liberating 7% and 33% more xylose than wheat-straw, respectively. Although containing high concentrations of fermentable glucose, the fermentation performance of the feedstocks generated after enzymatic saccharification was not evaluated.

3.3. Liberation of acetic acid from the pre-treatment process

Regardless of the LCM or pre-treatment employed, relatively high concentrations of acetic acid (30-75 mM; Fig. 2A) were present in all samples which would have been high enough to affect yeast growth rates and reduce glucose consumption (Narendranath et al., 2001). In general, use of autohydrolytical pre-treatment liberated lower concentrations of acetic acid (30-35 mM) when compared to the hydrolysates from the same LCM using either the alkaline or acid pre-treatments (61-69 mM and 45-73 mM respectively) (Fig. 2A).

3.4. Presence of weak acid, furan, and phenolic based inhibitors in the pre-treatment generated hydrolysates

The concentrations of weak acids (i.e., p-coumaric, ferulic, and formic acid), furans (i.e., HMF and furfural) and additional phenolic compounds (i.e., vanillin and syringaldehyde) present in hydrolysates after pre-treatment of LCM was measured. Very low concentrations of these compounds were detected in hydrolysates generated using alkaline (5% NaOH at 50°C) pre-treatment with the exception of syringaldehyde in hydrolysates from Miscanthus and wheat. Presence of 10 mM syringaldehyde has been observed to reduce ethanol productivity by approximately 30% when compared with unstressed controls without the compound present (Taherzadeh and Karimi, 2008). In the present study, 4 mM syringaldehyde was detected in hydrolysates derived from Miscanthus and 2 mM in hydrolysates derived from wheat (Fig. 2B).

3.5. Metabolic profiling of yeast cultured in pre-treatment generated hydrolysates (supplemented with glucose)

The PM was used for the metabolic profiling of the different pre-treatment generated hydrolysates (Fig. 3). The hydrolysates were supplemented with glucose to ensure a suitable carbon source was present (to avoid any starvation-induced effects) and any effects of the presence of the inhibitors could then be accurately determined. The starting pH of the
hydrolysates was adjusted to pH 5, as typical fermentations start at this pH and then subsequently typically drop to ca. pH 4.1 (Coote and Kirsop, 1976). The pH adjustment was problematic for the hydrolysates derived from the alkaline pre-treatment system as the pH (post pre-treatment) was ca. pH 14. As such, the adjustment down to pH 5 resulted in a significant increase in viscosity (significant effects on the colloidal stability) which made it subsequently problematic to work with from a practical point. Alternatively, the pH of acid and autohydrolytical pre-treatment generated hydrolysates was pH 2 and pH 6, respectively, and as such, the pH adjustment of these hydrolysates did not cause a fundamental change in viscosity or physical properties of the media.

Yeast metabolic output when cultured in hydrolysates derived from the alkaline pre-treatment system was severely reduced when compared to controls containing an identical quantity of glucose but no inhibitors (Fig. 4A). This was possibly due to the buffering that was required to adjust the pH down to pH 5 rather than the presence of syringaldehyde. There is also the possibility that alkaline pre-treatments generate hydrolysates had reduced nitrogen levels (in particular reduced free amino nitrogen or FAN levels) when compared with other pre-treatments. Overall, yeast metabolic output was shown to be higher when using acid or autohydrolytical pre-treatment derived hydrolysates than those derived from the use of alkaline pre-treatment (Figs. 4B and 4C). Additionally
wheat, willow, and Miscanthus. Plates were incubated at 30°C and read for 50 h, under anaerobic conditions: (A) Metabolic output (redox signal intensity) for hydrolysates derived from wheat-straw and Miscanthus using NaOH as a pre-treatment method, (B) Metabolic output (redox signal intensity) for hydrolysates derived from Miscanthus using NaOH as a pre-treatment method, and (C) Metabolic output (redox signal intensity) for hydrolysates from wheat, willow, and Miscanthus using hydrothermal methods (220°C) as a pre-treatment. Data are representative of triplicate values with standard deviation shown.

The fermentation performance of S. cerevisiaeNCYC292 was evaluated using the pre-treatment generated hydrolysate. This hydrolysate containing pentose sugars and inhibitory compounds was derived from the acid pre-treatment of wheat-straw and supplemented with 4% glucose (included as a useable carbon source). This was then compared with the fermentation performance of the same yeast strain when using just YPD media (also containing 4% glucose). The use of the acid pre-treatment paired with wheat-straw was chosen as the model system for further investigation due to a compromise between various factors and practical constraints. Wheat straw was chosen as the LCM biomass as use of this cereal straw has been highlighted as a potential energy crop within the UK constraints. Wheat straw was chosen as the LCM biomass as use of this cereal straw has been highlighted as a potential energy crop within the UK constraints. Wheat straw was chosen as the LCM biomass as use of this cereal straw has been highlighted as a potential energy crop within the UK constraints.
fermentation profiles when using 4% YPD as a control (Fig. 5B). However, during the initial stages of the fermentations, there was an approximate 2 h delay (extended yeast lag phase) between the test fermentation vessels (the pre-treatment derived hydrolysates) and the control fermentation vessels. However, even with the extended lag phase, all fermentations were still completed (attenuated) within 16 h. Quantification of ethanol concentrations produced indicated there was a ca. 36% conversion of glucose into ethanol from the hydrolysates which compares with the 51% theoretical maximum for the stoichiometric conversion of glucose into ethanol. Hydrolysates derived from the acid pre-treatment of wheat-straw were shown to contain acetic acid and furfural as the principal inhibitory compounds (Figs. 2A and 2B). Through the measurement of the effect of these inhibitors on yeast metabolic output, it was observed that acetic acid and furfural both individually reduced yeast metabolic output when compared with controls (Figs. 5C and 5D). Assays were all buffered to pH 5 prior to the start to mimic conditions present at the beginning of fermentation (Verduyn et al., 1990). Therefore, external pH-derived effects could be discounted and the deleterious effects of the inhibitors confirmed.

Fig.5. (A) Phenotypic microarray for *S. cerevisiae* (NCYC 2592) on hydrolysates derived from wheat using NaOH, H$_2$SO$_4$, and hydrothermal pre-treatment methods (200°C). (B) Fermentation kinetics analysis of *S. cerevisiae* NCYC 2592 using mini-fermenters on 4% (w/v) glucose or hydrolysate derived from wheat using acid hydrolysis as a pre-treatment spiked with glucose to give a final glucose concentration of 4% (w/v), (C) Effect of 25-50 mM acetic acid on yeast metabolic output, and (D) Effect of 1-10 mM furfural on yeast metabolic output. Data are representative of triplicate values with standard deviation shown.
4. Conclusions

Acid pre-treatment (1% H$_2$SO$_4$ at 121°C) was concluded to be optimal system (in terms of sugar liberation, inhibitor generation, and fermentability) when compared to the alkaline (5% NaOH at 50°C) or autohydrolytical (200°C) pre-treatment systems. Whilst alkaline pre-treatment was shown to enhance the enzymatic saccharification yields more than the other pre-treatments (and also the generated hydrolysate had the lowest concentration of inhibitors present), the hydrolysate showed poor fermentability. The fermentability of the hydrolysates was determined using a phenotypic microarray (PM) to measure yeast metabolic activity. The PM provided a rapid, high-throughput screening tool to access fermentation performance and could be used to evaluate which pre-treatment systems where optimal for different lignocellulosic biomass.

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