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Development of a phenotypic assay for characterisation of ethanologenic yeast strain sensitivity to inhibitors released from lignocellulosic feedstocks

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Abstract Inhibitors released by the breakdown of plant cell walls prevent efficient conversion of sugar into ethanol. The aim of this study was to develop a fast and reliable inhibitor sensitivity assay for ethanologenic yeast strains. The assay comprised bespoke 96-well plates containing inhibitors in isolation or combination in a format that was compatible with the Phenotypic Microarray Omnilog reader (Biolog, hayward, CA, USA). A redox reporter within the assay permits analysis of inhibitor sensitivity in aerobic and/or anaerobic conditions. Results from the assay were verified using growth on spot plates and tolerance assays in which maintenance of viability was assessed. The assay allows for individual and synergistic effects of inhibitors to be determined. It was observed that the presence of both acetic and formic acid significantly inhibited the yeast strains assessed, although this impact could be partially mitigated by buffering to neutral pH. *Scheffersomyces stipitis*, *Candida* spp., and *Pichia guilliermondii* demonstrated increased sensitivity to short chain weak acids at concentrations typically present in lignocellulosic hydrolysates. *S. cerevisiae* exhibited robustness to short chain weak acids at these concentrations. However, *S. stipitis*, *Candida* spp., and *P. guilliermondii* displayed increased tolerance to HMF when compared to that observed for *S. cerevisiae*. The results demonstrate that the phenotypic microarray assay developed in the current study is a valuable tool that can be used to identify yeast strains with desirable resistance to inhibitory compounds found in lignocellulosic hydrolysates.

Keywords Phenotypic microarray · Yeast · Bespoke · Inhibitory compounds · Bioenergy

Abbreviations

LCM	Lignocellulosic material
HMF	Hydroxymethylfurfural
PM	Phenotypic microarray
YPD	Yeast peptone broth
YNB	Yeast nitrogen base
OD	Optical density

Introduction

Fossil fuels, such as petroleum, coal, and natural gas, currently supply the majority of energy required to meet global energy demand. In 2008, it was estimated that 40 % of the total energy used in the USA was supplied by petroleum, 23 % by coal, and 23 % by natural gas with total fossil fuel utilisation accounting for 86 % of the total [20]. Depletion of conventional resources for generating energy, such as coal and crude oil, has been predicted to occur by 2050 [60]. These factors have stimulated interest in converting waste organic biomass into bioethanol as a potential renewable energy source [50].

Lignocellulosic material (LCM) as a biomass for bioethanol production is widely available, relatively inexpensive, and can be non-competitive with food production. The typical conversion of lignocellulose to ethanol consists of: (1)

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pre-treatment of plant biomass to permit effective hydrolysis by enzymes, (2) hydrolysis to yield sugars for fermentation, and finally (3) fermentation of sugars into ethanol and distillation [4].

Hydrolysis may be conducted in one step by the use of concentrated acid, under severe conditions, although this is energy and chemically intensive. Chemical hydrolysis releases sugars rapidly but delivers poor overall yields and generates degradation products, which can potentially inhibit the fermentation process [54]. The nature and concentrations of degradation products formed during pre-treatment are dependent on the type of biomass source material and the type of pre-treatment processes employed. Sugar degradation products such as furfural and hydroxymethylfurfural (HMF) are formed from pentose under auto-hydrothermal or acid-hydrothermal conditions at relatively high temperatures (180–220 °C) [14, 33, 54, 65]. Acetic acid is ubiquitous in hydrolysates where hemicellulose and components of the plant cell wall have acetyl groups that can undergo hydrolysis [14, 54, 65]. Formic acid may be formed as a by-product of sugar and lignin breakdown, whereas levulinic acid is generated from the degradation of HMF [14, 33, 54]. The lignin component of the plant material is particularly problematic because inhibitors are generated during cleavage and solubilisation of the aromatic subunits, producing phenolic compounds including ferulic acid, syringaldehyde, vanillin and vanillic acid, all of which are potential inhibitors of the fermentation process [14, 33, 54].

The precise mode of action for many of the inhibitors has yet to be fully determined [38]. Weak acid stress is induced when acetic, formic, or levulinic acid is liberated from LCMs; they inhibit yeast fermentations, reducing both growth and ethanol production. The inhibitory effects of weak acids have been linked to intracellular anion accumulation leading to reduced cytosolic pH, which in turn affects enzyme kinetics [47]. Pre-treatment processes also release phenolic compounds such as vanillin, and these compounds have demonstrated inhibitory effects on yeast cells [18, 19].

Strain selection for the production of ethanol from LCM-derived sugars has traditionally involved the use of several assays based on cell growth and division, and maintenance of viability in stress tests and fermentation analyses [6, 61]. Whilst very useful, these approaches are time consuming and interpretations can be subjective [16]. The phenotypic microarray (PM), developed by Bochner and colleagues [10, 12], provides an analogous two-dimensional array technology for simultaneous analysis of live yeast cell populations in a 96-well microtitre plate format. PM technology is a fully automated computer-linked technique based on a colourimetric assay that is monitored using an automated microtitre plate scanning spectrophotometer. The assay monitors' reduction of a redox-sensitive tetrazolium dye was proposed to detect cellular respiration [9, 11, 40]. Plate media arrays are commercially available, but none of these provide the potential to assess for key phenotypes required for LCM bioethanol production.

Currently, commercial PM arrays utilise pre-hydrated PM plates that allow for screening of up to 2,000 test substances [11]. These plates have been used to identify desirable phenotypic traits for fungi used in bio-processing [41] and ethanologenic bacterium *Zymomonas mobilis* ZM4 [8] but not for yeast strains to be used in biofuel fermentations. Use of bioscreens have been successfully employed for researching yeast performance in hydrolysates [64]; however, use of PM technology and Omnilog allows for up to 50 plates to be run at any time. PM assays do rely on detection of a colour development so assays with dark medium such as hydrolysates or wort could have better resolution in a plate reader or bioscreen C analyser. PM technology has not been used to assess the capacity of yeast strains to tolerate either stresses involved in biofuel fermentation or inhibitors typically present in LCM hydrolysates.

In this article, we describe a novel use of PM technology in which bespoke plates have been developed to permit screening of strains for tolerance to inhibitors typically generated in LCM deconstruction. In addition, a method by which aerobic and anaerobic conditions can be simultaneously assessed has been developed. This screening via PM technology was verified using both assessments of growth on spot plates and viability assays following inhibitor challenge. *Saccharomyces cerevisiae*, *Scheffersomyces stipitis*, *Candida shehatae*, *Candida succiphila*, and *Pichia guilliermondii* strains were screened for their inhibitor tolerance; these strains were selected because of their potential to convert hexose, and in some cases pentose, sugars into ethanol [1, 35, 52, 61, 62]. The concentrations of inhibitors deployed were based on a model hydrothermal treatment of wheat straw at a range of reaction temperatures. Additionally, we have analysed inhibitor tolerance under aerobic and anaerobic conditions and determined the impact of pH on the toxicity of the inhibitory compounds.

Materials and methods

Yeast strains and growth conditions

Saccharomyces cerevisiae NCYC 2592 and LAL7 (obtained from Lallemand Inc.), *C. shehatae* NCYC 2389, *C. succiphila* NCYC 1403, *P. guilliermondii* NCYC 443. and *S. stipitis* NCYC1541 were utilised in this study. All strains were 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 on 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.

Raw materials and model inhibitors

Model inhibitor compounds were supplied by Sigma (Dorset, UK) including acetic, formic, levulinic, coumaric, furoic, and ferulic acids, furfural, hydroxymethylfurfural (HMF), and vanillin; other chemicals were standard laboratory reagents. Wheat straw was harvested at the University of Nottingham.

Spot plate assays

Spot plate tests were performed according to Homann et al. [25] with modifications. Cells were grown overnight in yeast peptone broth (YPD) at 30 °C with orbital shaking at 150 rpm. One millilitre of culture ($OD_{600} = 1.0$) was centrifuged for 2 min at 17,000*g* in a swinging rotor centrifuge operated at 4 °C. The resulting pellet was washed three times using sterile distilled water and re-suspended in 100 µl of sterile distilled water. Next, the re-suspended cells were diluted to an initial OD_{600} of 1, serially diluted, and a 5-µl aliquot from each dilution was spotted onto agar plates (0.67 % YNB + 6 % glucose) containing various concentrations of formic acid as appropriate.

Viability assays

Viability assays were determined by growing cells in 50 ml YPD broth at 30 °C until the cells reached mid-exponential phase as measured by an OD_{600} reading between 0.4 and 0.6. The appropriate inhibitory compound was then added and the cell/inhibitor mixture was incubated aerobically for 15 min at 30 °C in an orbital shaker at 180 rpm. After incubation, OD was re-assessed and the cells were then diluted in fresh medium to 10^4 cells/ml, and 10 µl was plated in triplicate on YPD plates. Colonies were enumerated to obtain viable counts after 3 days at 30 °C. All spot tests and viability studies were performed in triplicate.

Model hydrothermal pretreatment of wheat straw

Dry wheat straw (2 g) was mixed with 8 ml demineralised water in stainless-steel tube reactors. These were sealed and held in an air-circulating oven, set at different temperatures for each reaction and timed for a period of 30 min from the point of insertion. The final temperature achieved during the time period was measured separately using a thermocouple inserted into a blank tube containing glycerol. At the end of the time period, the tubes were cooled under cold running water and opened, and the contents were then steeped with an additional 12 ml of water for 30 min, giving a 10:1 equivalent liquor-to-solid ratio. The hydrolysate liquor containing soluble products was then separated by vacuum filtration through Whatman GFA grade glass filter paper.

Detection of weak acids present in the pre-treatment hydrolysate

For the analysis of acetic and formic acid by HPLC, an aliquot of the hydrolysate (20 µl) was injected onto a 250 × 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.

Detection of furans and phenolics present in the pre-treatment hydrolysate

For the analysis of furan and phenolic inhibitors by HPLC, an aliquot of the hydrolysate (10 µl) was injected onto a 250 × 4.6-mm Techsphere C18 column (Capital HPLC Ltd., Broxburn, UK). The compounds were eluted with a gradient of methanol in 1 % aqueous acetic acid, delivered by two PU1580 pumps (Jasco). The methanol concentration was increased from 20 to 50 % over 30 min to elute the compounds of interest, purged with 100 % methanol for 2 min and then re-equilibrated for 10 min with 20 % methanol prior to the next injection. The flow rate was 1 ml/min and compounds were detected at 270 nm using a Spectro Monitor 3000 UV detector. The amount of each compound was determined by peak area comparison (Azur software) with authentic standards.

Phenotypic microarray analysis

Biolog growth medium was prepared using 0.67 % (w/v) yeast nitrogen base (YNB) supplemented with 6 % (w/v) glucose, 2.6 µl of yeast nutrient supplement mixture (NSx48- 24 mM Adenine-HCl, 4.8 mM L-histidine HCl monohydrate, 48 mM L-leucine, 24 mM L-lysine-HCl, 12 mM L-methionine, 12 mM L-tryptophan, and 14.4 mM uracil), and 0.2 µl of dye D (Biolog, Hayward, CA, USA). Final volume was made up to 30 µl using reverse osmosis (RO) sterile distilled water and aliquoted to individual wells with varying concentrations of appropriate inhibitors. Stock solutions (1 M) of aliphatic weak acids such as acetic, formic, and levulinic acids were prepared using RO sterile water; however, aromatic weak acids such as coumaric, ferulic, and furoic acids had to be prepared in

100 % ethanol. Furfural, HMF, and vanillin were prepared as 1 M stock solutions in 100 % ethanol. For assays utilising xylose, 6 % xylose (w/v) replaced glucose as the carbon source.

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 section of a fresh YPD plate 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 % (~5 × 10^6 cells/ml) using a Biolog turbidimeter (Biolog).

Cell suspensions for the inoculums were prepared by mixing 125 µl of the above cells with IFY buffer™ (Biolog) and the final volume 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™System address) with an anaerobic indicator (Oxoid, Basingstoke, UK) and the plates were placed inside PM gas bags (Biolog). 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 reflecting cell growth and dye conversion. Dye reduction, which reflects metabolic activity of cells, has been defined here as the redox signal intensity. After completion of the run, the signal data were 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.

The pH of media containing inhibitory compounds was measured, control without inhibitors was pH 6.5, and addition of furans or phenolic compounds had no effect on starting pH. Presence of weak acids reduced the starting pH as follows: 10 mM acetic acid pH 5, 25 mM acetic acid pH 4.7, 50 mM acetic acid pH 3.7, 75 mM acetic acid pH 3.4 and 100 mM acetic acid pH 3.2, 5 mM formic acid pH 3.8, 10 mM formic acid pH 3.1, 15 mM formic acid pH 2.9, and 20 mM formic acid pH 2.7; 10 mM coumaric, furoic, levulinic, or ferulic acid reduced the pH to 5.1, and 50 mM to pH 3.4.

Statistical analysis

Data derived from phenotypic microarrays were analysed for analysis of variance (ANOVA) using ezANOVA (http://www.cabiatl.com/mricro/ezanova/#defs), a free for use online statistical programme with statistical significance signified by use of *, *0.05 % significant, **0.01 % significant, and ***0.001 % significant.

Measurement of yeast growth

Yeast growth under identical growth conditions as for PM assays was monitored for 50 h with a reading every 15 min using a Tecan (Mannedorf, Switzerland) Infinite M200 Pro plate reader at 30 °C for 50 h. The assay was performed in triplicate and an average reading was plotted.

Glucose utilisation

Fermentation was conducted in 180-ml mini fermentation vessels (FV). Hydrolysates were made with 60 g/l (w/v) glucose and 6.7 g/l (w/v) minimal medium (YNB, yeast nitrogen base). Cryopreserved yeast colonies were streaked onto YPD plates and incubated at 30 °C for 48 h. Representative colonies were grown in 250-ml conical flasks containing 100 ml of YPD broth in an orbital shaker at 30 °C for 24 h. Cells were harvested and washed three times with sterile RO water and the pellet re-suspended in 5 ml of sterile water.

Cells for inoculation were prepared in 20 ml of hydrolysate, with transmittance adjusted to 62 % using the Biolog turbidometer. Hydrolysate in the mini FVs was inoculated with 4.16 ml of cells and adjusted to 120 ml with hydrolysate. Aerobic mini fermentations used a foam bung and tin foil to cover the mini FVs.

Mini fermentations were conducted statically at 30 °C and samples were taken aseptically using a 5-ml syringe and needle. Three replicate samples taken in duplicate were frozen at −20 °C until needed. When required, samples were allowed to thaw on the bench, filtered using a Ministart high flow 0.2-µm filter (Sartorius scientific, Goettingen, Germany), and placed in HPLC Chromacol vials (Chromacol, Welwyn, UK).

Detection of glucose and ethanol from FV experiments via HPLC

Glucose and ethanol concentrations were quantified by HPLC. The HPLC system included a Jasco AS-2055 Intelligent auto sampler (Jasco, Tokyo, Japan) and a Jasco PU-1580 Intelligent pump (Jasco). The chromatographic separation was performed on a Rezex ROA H^+ organic acid column, 5 µm, 7.8 mm × 300 mm (Phenomenex, Macclesfield, UK), at ambient temperature. The mobile phase was 0.005N H_2SO_4 with a flow rate of 0.5 ml/min. For detection a Jasco RI-2031 Intelligent refractive index detector (Jasco) was employed. Data acquisition was via the Azur software (version 4.6.0.0, Datalys, St Martin D'heres, France) and concentrations were determined by peak area comparison

with injections of authentic standards. The injected volume was 10 µl and analysis was completed in 28 min. All chemicals used were analytical grade (>95 % purity, Sigma-Aldrich, UK).

Results

Development of a phenotypic microarray assay for the impact of inhibitory compounds on metabolic output of ethanologenic yeast spp.

Using traditional yeast methodologies, such as spot plates, yeast strains have been identified as candidates for the production of ethanol from sugars derived from LCMs [14, 15]; however, the process is relatively slow, time consuming and subjective [16, 17].

The current study involved the development of bespoke phenotypic microarray (PM) plates in which the sensitivity of yeast strains to a range of inhibitory compounds was assessed. In addition the assessment of inhibitor tolerance in differing gaseous conditions typically experienced during fermentation was also demonstrated. This study examined the effect of inhibitory compounds that are typically released by the physiochemical decomposition of the cell wall; however, it is proposed that the assay could be extended to study the effect of any soluble relatively non-coloured compound on microbial metabolic output.

Metabolic output of *S. cerevisiae* NCYC 2592 as measured by the phenotypic microarray assay correlated with a measurable depletion of glucose and production of ethanol under similar conditions during fermentation (Fig. 1a, b).

The presence of formic acid (0–20 mM) reduced the rate of metabolic output (as measured by an increase in redox signal intensity) in *S. cerevisiae* (NCYC 2592) and *P. guilliermondii* (NCYC 443) when compared with controls (Fig. 1c, d). Presence of ethanol used as an organic solvent for coumaric acid, ferulic acid, furoic acid, HMF, and furfural had no effect on redox signal intensity when compared with redox signal curves containing no ethanol (data not shown).

Presence of 10 mM formic acid reduced yeast growth on solid agar (Fig. 1e, F) correlating with the reduction in metabolic output measured by redox signal intensity for *S. cerevisiae* NCYC 2592 and *P. guilliermondii* NCYC 443.

To further validate the observations from the PM assay, the effect of inhibitors on yeast growth was assessed using a plate reader in the same media and growth conditions applied within the PM assay. It was observed that metabolic profiles and growth curves appeared to correlate in the absence or presence of either acetic acid or furfural (Fig. 2a–d). The presence of inhibitory compounds slowed the conversion of sugar into biomass, and the extent to which this occurred was inhibitor and concentration dependent (Fig. 2b–d).

Data from phenotypic microarrays using a redox sensitive dye to measure metabolic output correlated well with yeast performance as measured by other techniques. An increase in metabolic output measured by redox signal intensity correlated with depletion of glucose and production of ethanol measured during fermentation under similar conditions (Fig. 1a, b). Reduction in metabolic output compared with metabolic output under control conditions caused by the presence of inhibitory compounds correlated with a reduction in yeast growth, giving us confidence in the robustness and reproducibility of the PM assay. Use of specialist equipment (Omnilog, Biolog) gives scope for the measurement of up to 50 microarray plates at any given time; this compares favourably with the standard one plate per run setup for standard plate readers.

Use of phenotypic microarray assay to determine ethanologenic yeast tolerance to inhibitory compounds

Because of the provided expected data for the Crabtree-positive yeast *S. cerevisiae*, other potential ethanologenic yeast species were also assessed for their tolerance to a range of inhibitory compounds. Four Crabtree-negative yeasts were selected as potential ethanologens: *S. stipitis* [45], *C. shehatae* [2], *C. succiphila*, which is a yeast with xylose and arabinose utilisation capabilities [53], and *P. guilliermondii* [21]. These were applied to the assay to validate the efficacy of the assay and to assess the tolerances of these yeasts to inhibitory compounds.

Tolerance of weak acids in yeast spp. when using a phenotypic microarray assay

Acetic acid and formic acid are released by hydrothermal pretreatment of straw (Table 1). The impact of these weak acids on *S. cerevisiae* has been investigated using PM plates [22]. Metabolic activity for *Candida*, *Pichia*, or *Scheffersomyces* yeast has not been previously reported. It was observed that 10 mM acetic acid had a pronounced effect on the metabolic activity of *S. stipitis*, *Candida* spp., and *P. guilliermondii* when compared to that observed for *S. cerevisiae* ($p = 0.001$). Indeed, metabolic output for *S. cerevisiae* was not inhibited until acetic acid levels reached 75 mM (Fig. 3a).

Assaying for the effect of formic acid on metabolic activity demonstrated that *S. stipitis*, *C. shehatae*, *C. succiphila*, and *P. guilliermondii* were inhibited by 5 mM, whereas *S. cerevisiae* was inhibited by 15 mM formic acid ($p = 0.001$) (Fig. 3b). Viability assays confirmed that *S. stipitis*, *Candida.* spp., and *P. guilliermondii* were more sensitive to acetic acid and formic acid than *S. cerevisiae* (Fig. 4a, b).

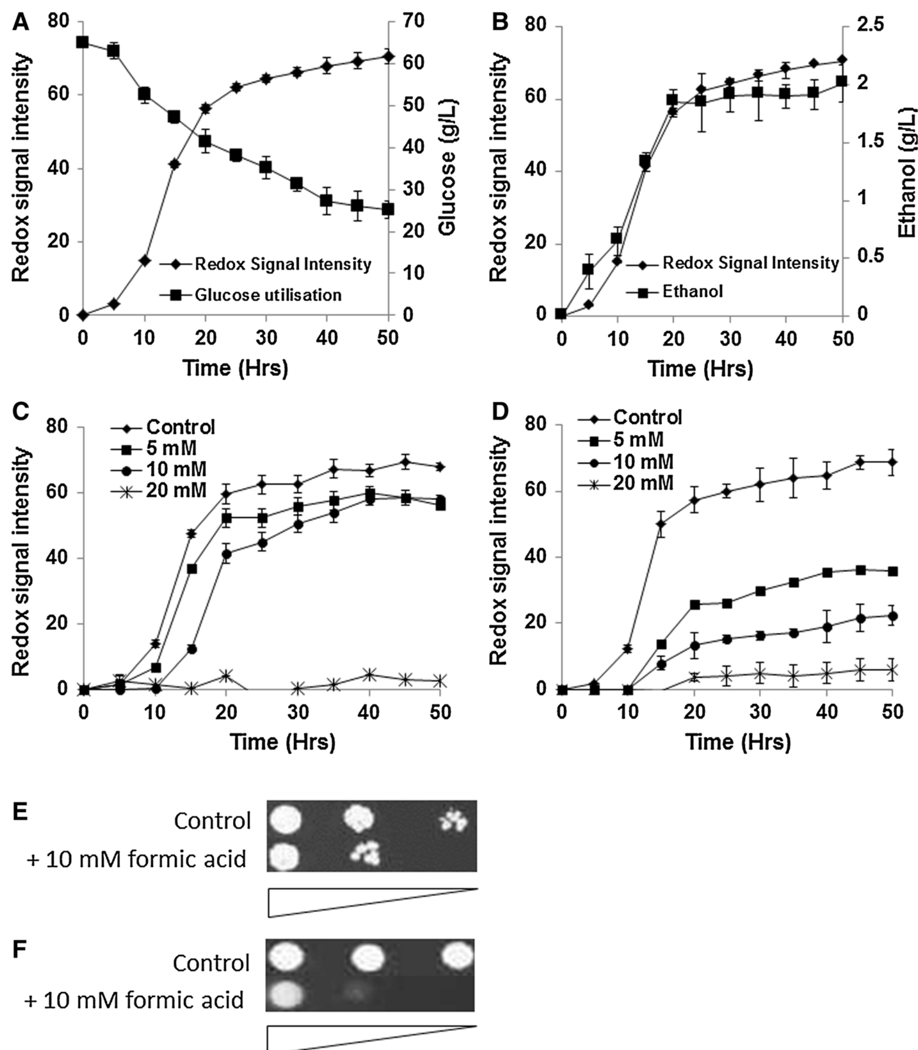

Fig. 1 Phenotypic microarray analysis (redox signal intensity) and performance in a fermentation for *S. cerevisiae* (NCYC 2592) and *P. guilliermondii* (NYCY 443) on media containing 0–20 mM formic acid. **a** Phenotypic microarray assays were incubated at 30 °C and read for 50 h, under aerobic conditions. *S. cerevisiae* 2,592 metabolic output in redox signal intensity, depletion of glucose from a 100-ml fermentation utilising 6 % glucose, 0.67 % YNB, under aerobic conditions at 30 °C for 50 h. **b** Phenotypic microarray assays were incubated at 30 °C and read for 50 h, under aerobic conditions. *S. cerevisiae* NCYC 2592 metabolic output in redox signal intensity and production of ethanol from a 100-ml fermentation utilising 6 % glucose, 0.67 % YNB, under aerobic conditions at 30 °C for 50 h. **c** Redox signal intensity for *S. cerevisiae* (NCYC 2592) 0–20 mM formic acid. **d** Redox signal intensity for *P. guilliermondii* (NCYC 443) 0–20 mM formic acid. Data representative of triplicate values with standard deviation shown. **e** Inhibition of metabolic output correlates with inhibition of growth, effect of 10 mM formic acid on growth on spot plates with *S. cerevisiae* NCYC 2592, and (**f**) effect of 10 mM formic acid on growth on spot plates with *P. guilliermondii* (NCYC 443)

Tolerance of yeast strains to furfural when using a phenotypic microarray assay

Presence of furfural had a pronounced effect on *C. shehatae* when compared with the other yeast assayed in this study. Furfural (5 mM) had a profound effect on metabolic activity and viability in *C. shehatae* when compared with *S. stipitis*, *S. cerevisiae* NCYC 2592, *C. succiphila*, and *P. guilliermondii* ($p = 0.001$) (Figs. 3c, 4g).

Candida spp. and *P. guilliermondii* were more tolerant to the inhibitory effect of HMF when compared with *S. cerevisiae*

Presence of HMF had an effect on redox signal intensity for *S. cerevisiae* NCYC 2592 when compared with *S. stipitis*, *Candida* spp., and *P. guilliermondii* ($p = 0.01$ in assays with 10 mM HMF and $p = 0.05$ in assays with 15 mM HMF) (Fig. 3d). Viability studies confirmed that *S.*

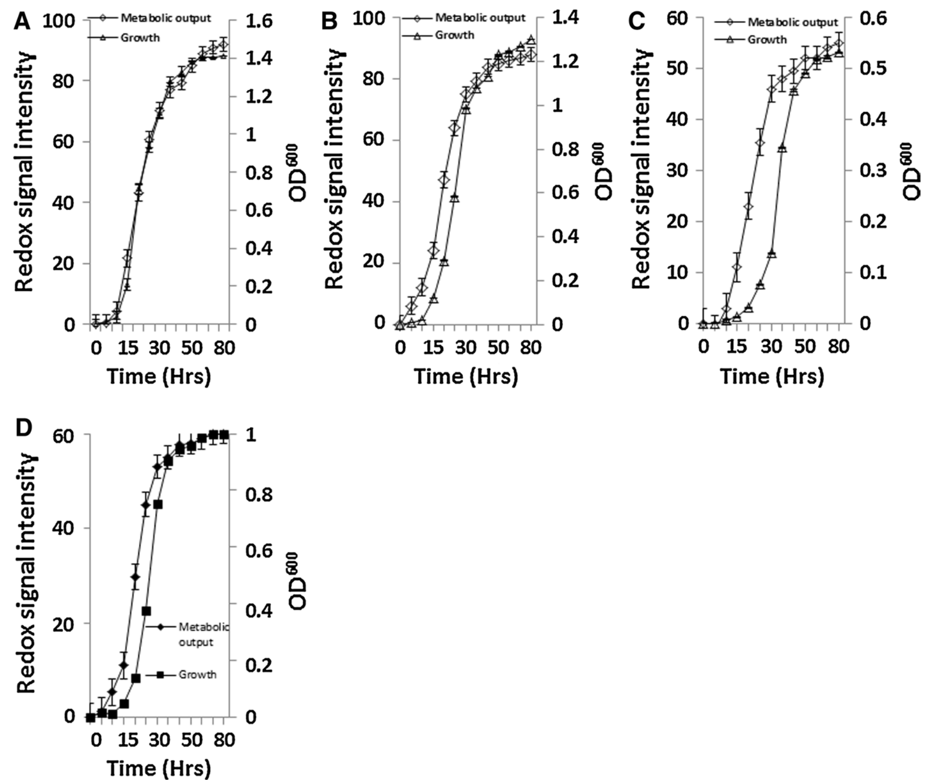

Fig. 2 Phenotypic microarray analysis and yeast growth for *S. cerevisiae* NCYC 2592 growth under control conditions and in the presence of inhibitory compounds. **a** Redox signal intensity and yeast growth under control conditions for *S. cerevisiae* NCYC 2592. **b** Redox signal intensity and yeast growth under control conditions for *S. cerevisiae* NCYC 2592 in the presence of 10 mM acetic acid. **c** Redox signal intensity and yeast growth under control conditions for *S. cerevisiae* NCYC 2592 in the presence of 50 mM acetic acid. **d** Redox signal intensity and yeast growth under control conditions for *S. cerevisiae* NCYC 2592 in the presence of 5 mM furfural. Data representative of triplicate values with standard deviation shown

Table 1 Composition of inhibitors released from wheat straw using different pre-treatment conditions

Compound (mM)	Temperature				
	130 °C	150 °C	170 °C	190 °C	210 °C
Acetic acid	6.6 ± 1.1	13.3 ± 2.2	21.6 ± 3.6	62.4 ± 10	89.93 ± 15
Formic acid	4.3 ± 0.3	5.4 ± 0.4	10.9 ± 0.8	32.6 ± 2.3	103.2 ± 7.2
Furfural	0.1 ± 0.02	0.2 ± 0.04	0.3 ± 0.06	1.04 ± 0.2	8.3 ± 1.7
HMF	0.08 ± 0.02	0.08 ± 0.02	0.08 ± 0.02	0.4 ± 0.08	0.8 ± 0.16
Vanillin	<0.1	<0.1	<0.1	<0.1	<0.1
Other weak acids	<0.1	<0.1	<0.1	<0.1	<0.1

Dry straw (2 g) was mixed with 8 ml demineralised water in stainless-steel tube reactors. These were sealed and held in an air-circulating oven, set at different temperatures for each reaction and timed for a period of 30 min from the point of insertion. The hydrolysate liquor containing soluble product was then separated by vacuum filtration through a Whatman GFA grade glass filter paper and measured via HPLC

cerevisiae strains were more sensitive to HMF than other yeast strains assayed in this study (Fig. 4h). Assays with other *S. cerevisiae* strains revealed that HMF sensitivity was species specific as other *S. cerevisiae* strains also display sensitivity to HMF when compared with other yeast (data not shown).

C. succiphila was more sensitive to the inhibitory effect of vanillin when compared with other yeasts

Phenolic compounds such as vanillin are released by the pre-treatment of LCMs (Table 1); vanillin had a pronounced effect on *C. succiphila* when compared with the other yeasts assayed in this study. Metabolic output and viability were profoundly affected for *C. succiphila* by 5 mM vanillin ($p = 0.01$), whereas *S. cerevisiae*, *S. stipitis*, *C. shehatae*, and *P. guilliermondii* were not inhibited until the vanillin concentration reached 15 mM (Figs. 3e, 4i).

Vanillin from pre-treatment of straw occurs at concentrations of less than 0.1 mM in LCM hydrolysates (Table 1); however, vanillin concentrations of up to 2 mM (430 mg/l) from spruce have been reported [30, 44]. The presence of 0.1 mM vanillin only had an effect on redox signal intensity on *C. succiphila* (Fig. 3e). There are no reports concerning the tolerance of *C. succiphila* to vanillin.

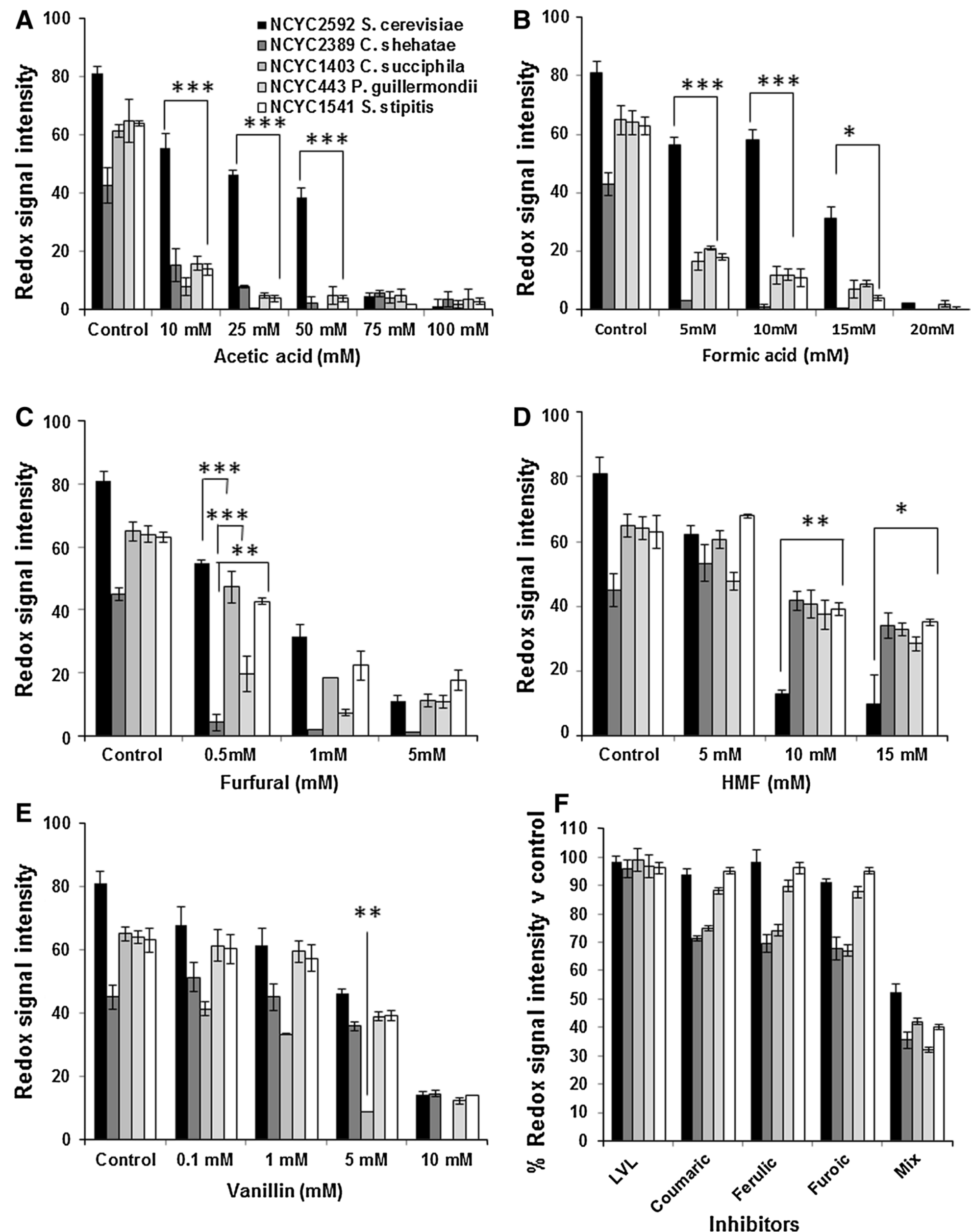

Fig. 3 The effect of inhibitory compounds on sugar utilisation expressed as redox signal intensity units on yeast spp. **a** Acetic acid 0–100 mM. **b** Formic acid 0–20 mM. **c** Furfural 0–5 mM, **d** HMF 0–15 mM, **e** vanillin 0–10 mM, and **f** weak acids (coumaric, levulinic, furoic, and ferulic acid), all 0.1 mM (data expressed as % of control signal intensity). NCYC 2592, *S. cerevisiae*; NCYC 2389, *C. shehatae*; NCYC 1403, *C. succiphila*; NCYC443, *P. guilliermondii*; NCYC1541, *S. stipitis*. Data representative of triplicate values with standard deviation shown

Presence of low abundance weak acids in pre-treatment hydrolysates was not inhibitory to yeast

Weak acids such as coumaric, levulinic, furoic, and ferulic have been reported to occur at concentrations of less than 0.1 mM in LCM hydrolysates (Table 1) [15, 33, 57]. When exposed to these acids individually at these concentrations, no detectable change in redox signal intensity could be determined for any yeast strains (Fig. 3f). However, when yeast strains were presented with a combination of these acids, a reduction in redox signal of 40–60 % was observed, indicating that even when present at low concentrations, weak acids act synergistically to inhibit yeast metabolic activity (Fig. 3f). At higher concentrations exposure to individual acids was observed to impair viability; however, there was little variation observed between yeast strains assayed for this response (Fig. 4c, d, e, f).

Tolerance to inhibitory compounds whilst using xylose as a sole carbon source in phenotypic microarray assay plates

Hydrolysates are composed of hexose and pentose sugars [57]. Assays using 6 % xylose as a sole carbon source with *S. stipitis*, a xylose-utilising yeast [1], revealed that this yeast exhibited similar tolerances to inhibitory compounds

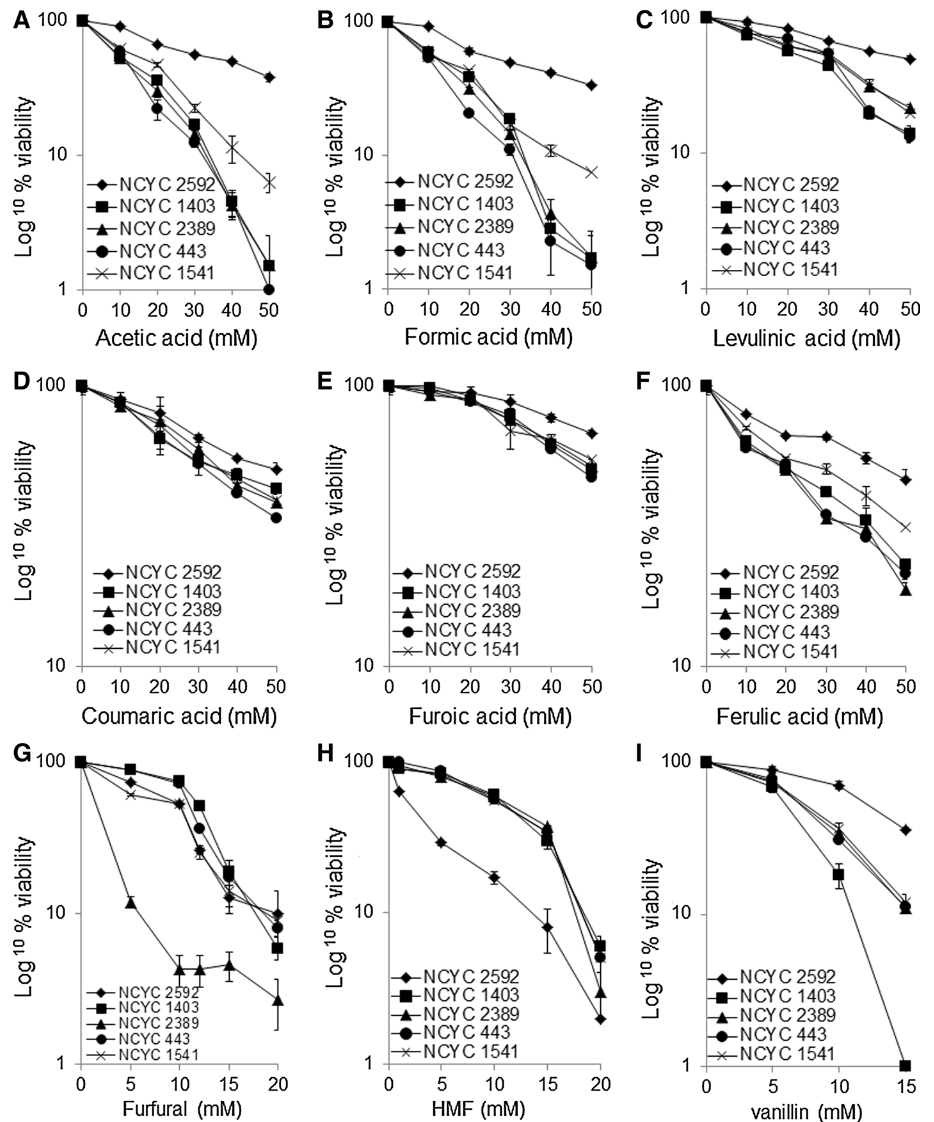

Fig. 4 The effect of inhibitory compounds on yeast viability. Viability to **a** acetic acid 0–50 mM, **b** formic acid 0–50 mM, **c** levulinic acid 0–50 mM, **d** coumaric acid 0–50 mM, **e** furoic acid 0–50 mM, **f** ferulic acid 0–50 mM, **g** furfural 0–20 mM, **h** HMF 0–20 mM, and **i** vanillin 0–15 mM. NCYC 2592, *S. cerevisiae*; NCYC 2389, *C. shehatae*; NCYC 1403, *C. succiphila*; NCYC443, *P. guilliermondii*; NCYC1541, *S. stipitis*. Data representative of triplicate values with standard deviation shown

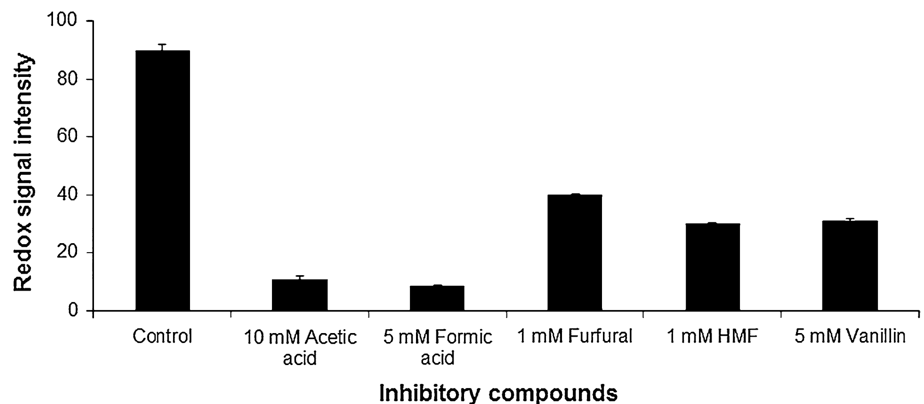

Fig. 5 The effect of inhibitory compounds on sugar utilisation expressed as redox signal intensity on *S. stipitis* utilising 6 % xylose and in the presence of 10 mM acetic acid, 5 mM formic acid, 1 mM furfural, 1 mM HMF, or 5 mM vanillin. Data representative of triplicate values with standard deviation shown

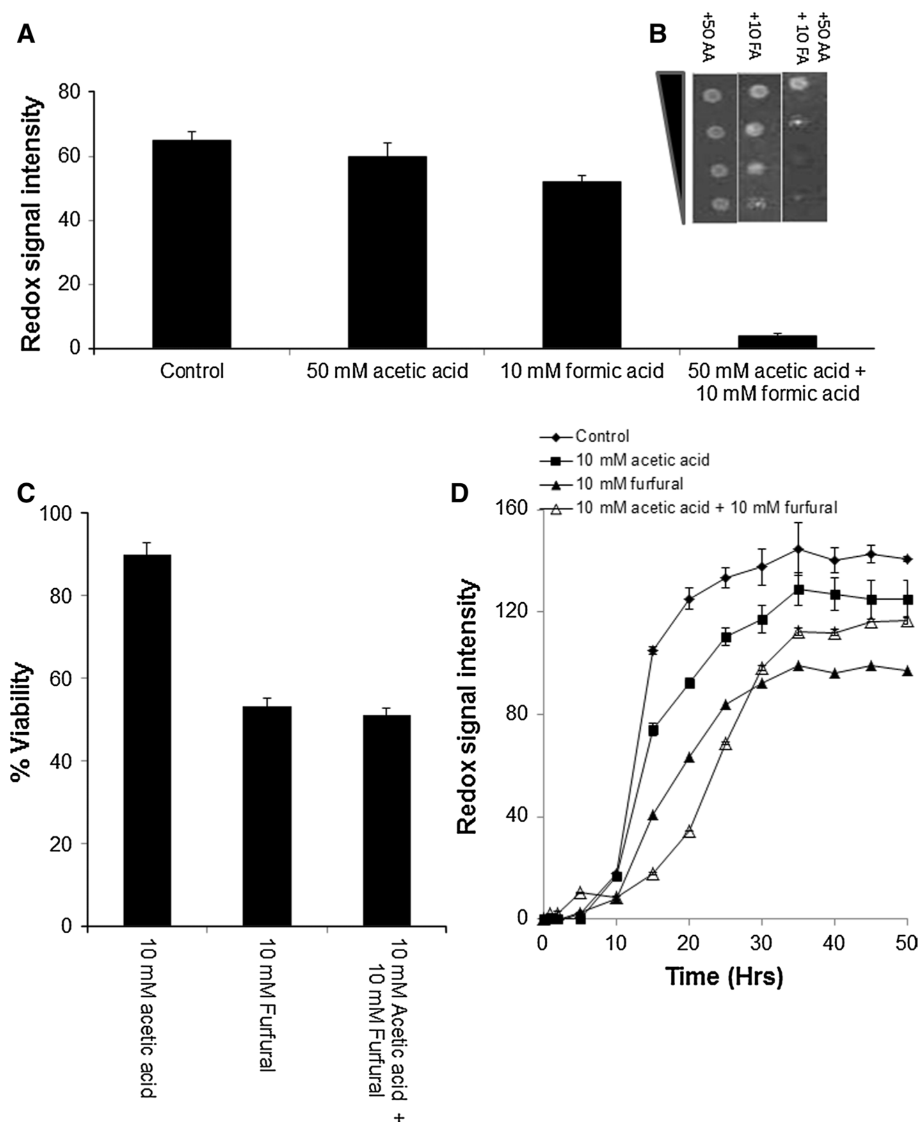

Fig. 6 Synergistic effect of weak acids on growth and sugar utilisation. **a** Sugar utilisation in the presence of 50 mM acetic acid, 10 mM formic acid, and 50 mM acetic acid with 10 mM formic acid combined. Data representative of triplicate values with standard deviation shown. **b** Spot plate assays (0.67 % YNB + 6 % glucose) containing 50 mM acetic acid, 10 mM formic acid, and 50 mM acetic with 10 mM formic acid, respectively. **c** Viability of *S. cerevisiae* NCYC 2592 in the presence of 10 mM acetic acid, 5 mM furfural, and 10 mM acetic acid with 5 mM furfural. **d** Sugar utilisation in the presence of 10 mM acetic acid, 5 mM furfural, and 10 mM acetic acid with 5 mM furfural

compared with assays with glucose as a sole carbon source (Figs. 3a–e, 5).

Inhibitors worked synergistically to inhibit redox signal intensity and yeast growth

The presence of more than one inhibitory compound has been reported to have a synergistic effect on yeast with consequent reduction in ethanol production [63]. Measuring metabolic output under inhibitory stress allows the synergistic effect of inhibitory compounds to be readily tested. The presence of 50 mM acetic acid or 10 mM formic acid demonstrated no detectable change in redox signal intensity on *S. cerevisiae* NCYC 2592 when compared with controls. However, there was a pronounced effect on redox signal intensity and growth in the presence of both weak acids (Fig. 6a, b). There was no observed effect on viability or redox signal intensity with furfural in combination with acetic acid (Fig. 6c, d).

Candida spp. and *P. guilliermondii* were sensitive to acidic conditions when compared with *S. cerevisiae*

Ethanologenic fermentation by *S. cerevisiae* from lignocellulosic material is usually carried out at a starting point of pH 5 to allow for an efficient fermentation [59]. Ethanologenic strains must be able to function across an appropriate pH range (pH 4–6).

Assaying for the effect of pH on metabolic activity, it was established that there was a significant reduction in signal intensity in *Candida* spp. and *P. guilliermondii* spp. under acetic acid stress at pHs 4–5 when compared with *S. cerevisiae* (Fig. 7a). *S. cerevisiae* grew on solid media (YPD) containing 20 mM acetic acid at pH 5; however,

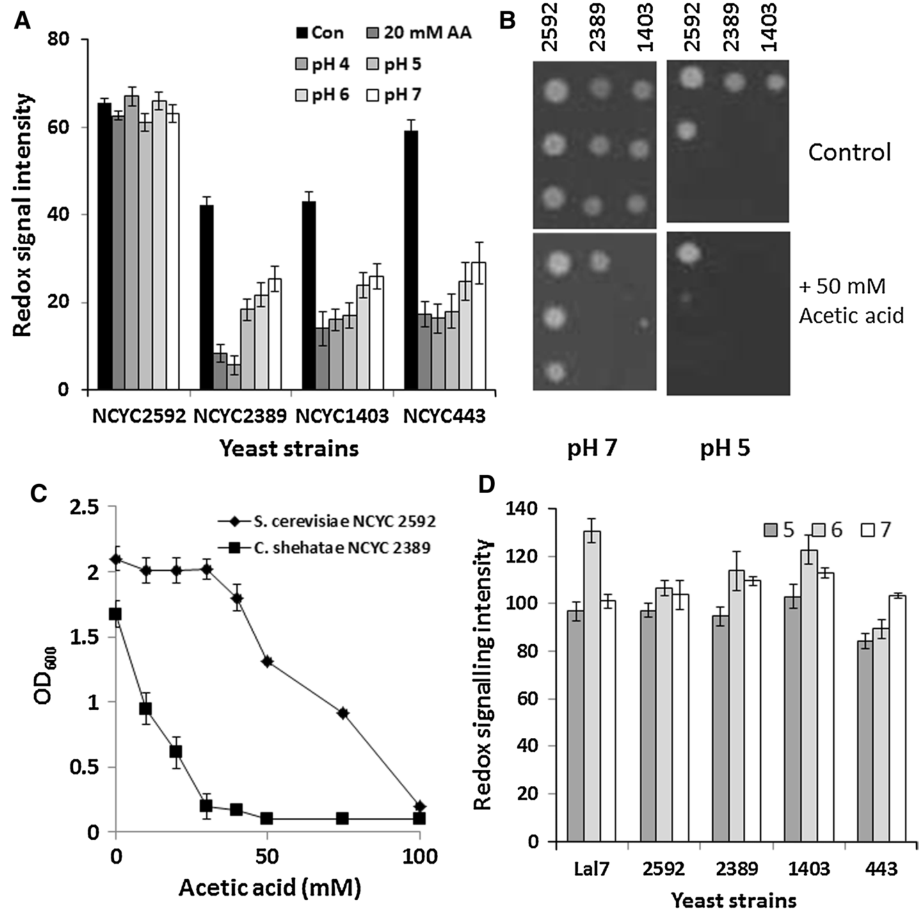

Fig. 7 Effect of pH on the toxicity of inhibitory compounds on yeast spp. **a** Effect of pH (pH 4–7) on sugar utilisation in yeast spp. stressed with 20 mM acetic acid. **b** Growth on solid media (YEPD) containing 50 mM acetic acid at pH 5 and pH 7. **c** Effect of acetic acid and pH on yeast growth measured by OD^{600}. **d** Effect of pH (pH 5–7) on sugar utilisation in the presence of 5 mM furfural. NCYC 2592, LAL7, *S. cerevisiae*; NCYC 2389, *C. shehatae*; NCYC 443, *P. guilliermondii*; NCYC 1403, *C. succiphila*. Data representative of triplicate values with standard deviation shown

growth of *Candida* spp. and *P. guilliermondii* was inhibited (Fig. 7b). Measuring growth (OD_{600} reading after 24 h) demonstrated that *S. cerevisiae* tolerated 50 mM acetic acid with a starting pH of 5; however, *C. shehatae* was sensitive to 30 mM acetic acid (Fig. 7c). There was no correlation between furfural toxicity and pH (Fig. 7d).

Inhibitory compounds were more toxic under anaerobic conditions when compared with aerobic conditions

Classic yeast fermentation is a process that begins in an aerobic environment but transitions to an anaerobic environment [32]; inhibitor toxicity was therefore assessed in the presence and absence of oxygen. To represent differences in redox signal intensities under aerobic and anaerobic conditions, data are shown as a percentile of redox signal intensity against the control and the presence of inhibitors. The setup for the phenotypic microarray is an aerobic one, although use of oxygen-absorbing pack conditions within the plate would best be described as micro-aerobic rather than anaerobic. A reduction in redox signal intensity occurred when acetic acid was present in an anaerobic as opposed to an aerobic environment, and this reduction was concentration dependent (Fig. 8a). In contrast, there was no detectable difference in redox signal intensity in the presence of furfural under aerobic or anaerobic conditions (Fig. 8b).

Discussion

The major components of LCM are cellulose, hemicellulose, and lignin, though the relative abundance of these is dependent on the LCM source [34]. Different processes have been developed for the deconstruction of plant cell wall material; these include weak acid, weak base, and steam explosion at a range of temperatures (130–210 °C) [56], and each method may generate inhibitors [34] though little has been published concerning means by which inhibitor formation can be restricted. However, the effect of inhibitors can be overcome by engineering strains for inhibitor tolerance [5, 29].

As a consequence current expectations are that ethanologenic yeast capable of converting sugars released from LCM into ethanol will need to be robust and able to withstand the inhibitors generated by the pre-treatment processes [39]. Therefore identification of yeast strains capable of tolerating high levels of single and/or mixed inhibitors

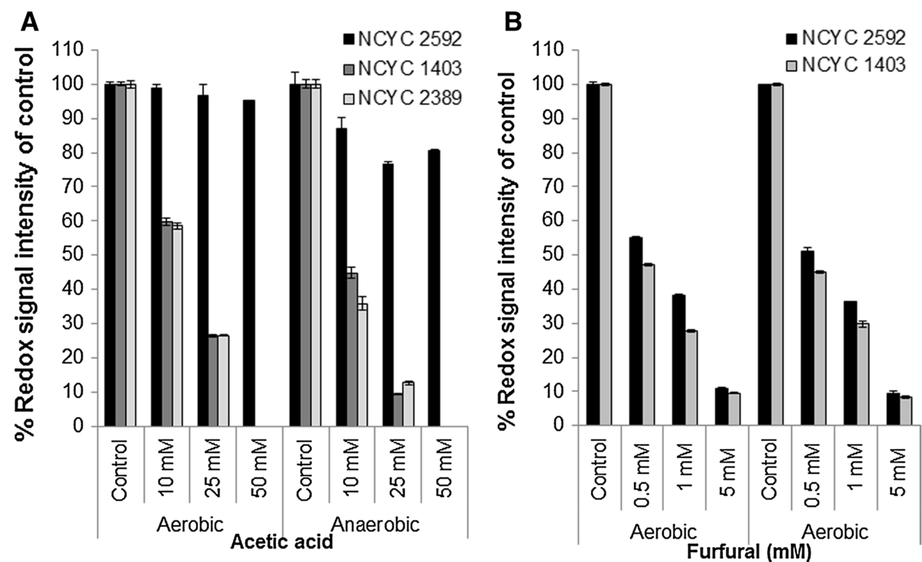

Fig. 8 Effect of oxygen on inhibitor toxicity. Effect of inhibitors on sugar utilisation under aerobic and anaerobic conditions as expressed as a percentage of unstressed controls: **a** 0–50 mM acetic acid, **b** 0–5 mM furfural. NCYC 2592, *S. cerevisiae*; NCYC 2389, *C. shehatae*; NCYC 1403, *C. succiphila*. Data representative of triplicate values with standard deviation shown

would be beneficial [17]. Previous strain selection methodologies have focussed on sugar utilisation and have been both time consuming and not conducive to screening multiple strains simultaneously [42].

Phenotypic microarray technology has been available for a number of years as a means of characterising yeast for nutritional requirements [28] or the optimal use of complex media [13]. However, this technology has not been used to screen yeast for tolerance to inhibitors released from LCM deconstruction. One reason for this omission is that all studies published to date have relied on the use of plates supplied by the manufacturers and these have not included plates for inhibitor screening.

In this study, we describe an assay that combines PM technology with bespoke plates specifically developed for the identification of strains with inhibitor resistance. We have displayed that cellular performance in micro assay volumes is analogous to performance in fermentations and correlates with sugar utilisation and growth. Use of bespoke plates described here has been used to investigate the role of proline in weak acid-stressed cells [22].

Metabolic activity was monitored using a redox reporter and relative activities expressed as signal intensity, which corresponded well with glucose utilisation and ethanol production in fermentations under similar conditions. The activity correlated to the depletion of glucose and in addition to yeast growth. In the presence of an inhibitory compound, a delay in signal intensity increase occurred, which was dependent on the concentration of the inhibitor and the yeast strain screened. Lag in signal intensity increase was deemed to indicate sensitivity to the inhibitor and dependent on the extent of the lag could be utilised to rank strains in terms of inhibitor tolerance. Metabolic activity correlated with yeast viability. Reduced metabolic activity in the presence of inhibitory compounds was echoed by a reduction in viability and appeared to be consistent for all yeast strains and inhibitory compounds measured in this article. Measuring yeast growth in the presence of inhibitory compounds also demonstrated a correlation between reduced metabolic activity (redox signal intensity) and growth. The presence of inhibitory compounds had an additional effect of increasing the delay between observed metabolic output and yeast growth.

Weak acids (acetic and formic acid) inhibited *S. stipitis*, *Candida* spp., and *P. guilliermondii* at concentrations previously reported to occur in LCM hydrolysates [3, 55]. In contrast, *S. cerevisiae* was observed to be more tolerant and this observation confirms previous reports that strains of this species can tolerate 60 mM acetic acid [24]. Acetic and formic acid induced reduction in metabolic activity as indicated by signal intensity for *S. stipitis*, *Candida* spp., and *P. guilliermondii* correlated with a loss of viability and a slow growth phenotype.

Sensitivity to 20–100 mM formic acid for *S. cerevisiae* has been previously reported [31] and supports the data presented in the current study. Tolerance of *P. guilliermondii* to formic acid has not been reported previously; however, growth in the closely related yeast *P. deserticola* is known to be inhibited by 20 mM formic acid [49] supporting the data presented in the current study.

Presence of longer chain weak acids had little effect on metabolic activity as indicated by redox signal intensity or viability at concentrations assayed. These data support previous studies in which it was demonstrated that levulinic acid and ferulic acid have little impact on *S. cerevisiae* [43] [15]; however, tolerance levels for *S. stipitis*, *Candida* spp. and *P. guilliermondii* have not been reported previously. Tolerance to furoic acid has been reported in *Trichosporon*

fermentas at concentrations analogous to those for yeast assayed in this study [27].

Presence of furan compounds is an unavoidable consequence of the use of temperature (>140 °C) to break LCM into fermentable sugars [56]; indeed, steam explosion methods (220 °C) may generate up to 8 mM furfural [7]. *C. shehatae* displayed sensitivity to furfural when compared to the other yeasts assessed. Previous studies have demonstrated that 20 mM furfural inhibits *S. cerevisiae* [46], with tolerance developing through upregulation of alcohol dehydrogenase genes [36]; constant exposure to furfural (10–40 mM) has been used to improve tolerance in *S. stipitis* with genes involved in glycolysis, pentose phosphate pathways, and tricarboxylic cycles all upregulated [23]. However, tolerance to this compound for other yeasts has not been reported.

HMF is a furanic compound liberated from LCM; *S. cerevisiae* was more sensitive to HMF than the other yeasts investigated. Although the reasons for this difference are not known, suggestions are that the ability of the yeast to modify HMF to 5-hydroxymethylfurfural alcohol is important [48]. There have been no previous reports concerning tolerance of *S. stipitis*, *Candida* spp., and *P. guilliermondii* to HMF.

Phenolic compounds such as vanillin are present at low concentrations in hydrolysates (<0.1 mM). At these concentrations vanillin had no detectable effect on metabolic activity or viability of *S. cerevisiae, S. stipitis, C. shehatae*, or *P. guilliermondii*. However, *C. succiphila* appeared to be particularly sensitive to this inhibitor. Sensitivity to higher concentrations of vanillin (10 mM) for *S. cerevisiae* and *P. guillermondii* has been previously reported [3, 18], supporting the data presented in this study.

Use of combinations of inhibitors enhanced the relative sensitivity of the yeast strains, irrespective of genus and species. A full study into the synergistic effect of inhibitors requires a structured approach as defined by Torres et al. [58] and this work is currently being pursued.

Conclusions

A method for the identification of yeast strains that are tolerant to LCM degradation inhibitors has been developed. The method utilised existing phenotype microarray technology with novel assay plates that will permit the simultaneous assessment of multiple strain sensitivity to individual and mixed inhibitors. The assay monitors metabolic activity using a redox reporter and this correlated well with viability and growth inhibitor assessments. Using the newly developed assay it was established that combined inhibitors were more toxic than individual inhibitor challenges, irrespective of the genus or strain of yeast assessed. It was also noted that the relative toxicity of inhibitors could be assessed. Finally non-*Saccharomyces* yeasts exhibited enhanced sensitivity to short chain weak acids such as acetic and formic acid when compared with *S. cerevisiae*, suggesting that the latter may be more suitable for conversion of LCM hydrolysates into ethanol. This observation has not been previously reported. Development of resistance to inhibitory compounds has centred around constant exposure of the yeast to the inhibitor [26, 37] or through selected expression of a key gene [51]. These screens would be greatly improved by the use of a quantifiable, reliable, and quick assessment of tolerance such as use of PM assays.

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Conflict of interest The authors declare that they have no competing interests and all authors have agreed to submit the paper to the Journal of Industrial Microbiology and Biotechnology.

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