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Phenotypic characterisation of *Saccharomyces* spp. for tolerance to 1-butanol

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Abstract Biofuels are expected to play a role in replacing crude oil as a liquid transportation fuel, and research into butanol has highlighted the importance of this alcohol as a fuel. Butanol has a higher energy density than ethanol, butanol–gasoline blends do not separate in the presence of water, and butanol is miscible with gasoline (Szulczyk, Int J Energy Environ 1(1):2876–2895, 40). *Saccharomyces cerevisiae* has been used as a fermentative organism in the biofuel industry producing ethanol from glucose derived from starchy plant material; however, it typically cannot tolerate butanol concentrations greater than 2 % (Luong, Biotechnol Bioeng 29 (2):242–248, 27). 90 *Saccharomyces* spp. strains were screened for tolerance to 1-butanol via a phenotypic microarray assay and we observed significant variation in response with the most tolerant strains (*S. cerevisiae* DBVPG1788, *S. cerevisiae* DBVPG6044 and *S. cerevisiae* YPS128) exhibiting tolerance to 4 % 1-butanol compared with *S. uvarum* and *S. castelli* strains, which were sensitive to 3 % 1-butanol. Response to butanol was confirmed using traditional yeast methodologies such as growth; it was observed that fermentations in the presence of butanol, when using strains with a tolerant background, were significantly faster. Assessing for genetic rationale for tolerance, it was observed that 1-butanol-tolerant strains, when compared with 1-butanol-sensitive strains, had an up-regulation of *RPN4*, a transcription factor which regulates proteasome genes. Analysing for the importance of *RPN4*, we observed that a Δ*rpn4* strain displayed a reduced rate of fermentation in the presence of 1-butanol when compared with the BY4741 background strain. This data will aid the development of breeding programmes to produce better strains for future bio-butanol production.

Keywords *Saccharomyces* spp. · 1-Butanol · Phenotypic microarray · Fermentation · qPCR

Introduction

Hydrocarbon fuels for transportation are currently derived from fossil-based crude oil and are not infinite resources. In 2008, 55 % of crude oil was used for transportation fuels; biofuels can be used in internal combustion engines making them attractive replacements for fossil transport fuel in the near future. Presently, the most widely used biofuel is ethanol derived from sugarcane, maize or wheat starch or the conversion of plant cell wall sugars.

Butanol is less hygroscopic and has a longer chain length than ethanol [12]. It can also be blended up to 85 % with fossil fuels compared with 10 % of ethanol due to engine requirements. Butanol has four isomers of which 1-butanol and isobutanol have been produced in yeast [10, 36]; isobutanol can also be converted into isobutylene or kerosene [9].

Clostridium acetobutylicum ATCC 824 produces 1-butanol; however, toxicity attributed to the chaotropic effect on the cell membrane is severe [5]. Wild-type *C. acetobutylicum* fermentations rarely manufacture more than

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13 g/L, a level generally considered to be at the toxic limit [2]; however, genetic modifications have improved tolerance to butanol [19, 20]. *Clostridium beijerinckii* can also manufacture 1-butanol from cornstarch [14] or a hemicellulosic hydrolysate from corn fibres [18].

Isobutanol can be produced naturally in yeast; its production consists of the anabolic synthesis of ketoisovalerate (an intermediate in valine biosynthesis) in the mitochondria before it is transported into the cytosol and converted into isobutanol using pyruvate decarboxylase and alcohol dehydrogenase enzymes [10]. Butanol production in unmodified yeast strains is around 0.16 mg isobutanol per gram of glucose; however, isobutanol production can be improved to around 2 mg per gram of glucose through the overexpression of enzymes which converts pyruvate into valine in the mitochondria before valine is further converted in the cytosol, preventing ethanol production [10]. Compartmentalisation of the Ehrlich pathway into the mitochondria converting valine into isobutanol, rather than the release of valine into the cytosol, increased the production 260-fold compared with the same enzymes in the cytosol which induced a 10 % increase [1].

Saccharomyces cerevisiae has been engineered to produce 1-butanol and/or isobutanol by substituting enzymes from *C. acetobutylicum* [39]. This approach increased butanol production up to 2.5 mg/L using galactose as the carbon source. Yeast strains have been engineered with improved carbon flux towards acetyl-CoA the precursor for 1-butanol production. This approach increased butanol titres during fermentation to 16 mg/L [23]. Alternate approaches such as the use of glycine as a carbon source have led to titres of 92 and 58 mg/L for butanol and isobutanol, respectively [7].

The endogenous activity of the Ehrlich pathway for production of higher alcohols can be enhanced by overexpressing 2-keto acid decarboxylase and alcohol dehydrogenase [21]. A recent work has highlighted that overexpression of *ILV2* (which catalyses the first step in the metabolism of valine), along with the insertion of a gene encoding enzymes catalysing the degradation of 2-ketoisovalerate (*KIVD* of *Lactococcus lactis*) in a Δ*pdc1* deletion strain, increased the final titre of isobutanol [22], preventing Ilvp2, Ilvp3 or Ilvp5 which are endogenous mitochondrial enzymes in the pathway from entering the mitochondria. This has been shown to increase isobutanol production to 15 mg/g glucose [8]. Conversely, compartmentalisation of the Ehrlich pathway into the mitochondria converting valine into isobutanol, rather than the release of valine into the cytosol, increased isobutanol production 260-fold compared with the same enzymes in the cytosol which induced a 10 % increase in isobutanol production [1]. Yeasts with extensive modifications to their cell wall integrity have been patented as 1-butanol-, 2-butanol- and isobutanol-tolerant strains. These strains have measurable improvement in growth in the presence of 1 % isobutanol [6].

The *Saccharomyces* spp. are yeasts specialised for utilisation of sugar into biomass and production of ethanol. The genus consists of seven closely related, but distinct species isolated from various geographical destinations: *S. cerevisiae, S. paradoxus, S. mikatae, S. kudriavzevii, S. arboricolus, S. uvarum* and *S. castelli* [28, 29, 41]. *S. cerevisiae* is the most common species used in wine, bread, ale beer and sake fermentations, although *S. uvarum* is also involved in lager beer, wine and cider fermentations [31, 32]. *S. paradoxus* has only once been associated with wine fermentation [33]. The other three species, *S. arboricolus, S. mikatae* and *S. kudriavzevii*, are wild isolates [30] and have never been associated with beverage fermentations.

Saccharomyces cerevisiae strains contain a significant amount of genetic variability as a consequence of human intervention and cross hybridisation [25]. Unlike *S. paradoxus* isolates which generally developed from one population, *S. cerevisiae* isolates are thus mosaics of two or more of the five clean populations known. These clean mosaic lineages of *S. cerevisiae* strains which display the same phylogenetic relationship across their entire genomes have been identified (Malaysia, West Africa, Wine European, Sake and North America) [25].

Previous studies have indicated that within *Saccharomyces* spp., *S. cerevisiae* strains can tolerate high ethanol concentrations [3]. We aimed to identify 1-butanol-tolerant yeast strains via phenotypic microarray analyses, measure performance in fermentations in the presence of 1-butanol and discover genes involved in 1-butanol stress response.

Materials and methods

Strains, media and growth conditions

Many of the *Saccharomyces* spp. strains used in this study have been previously described [24–26]. Additional isolates tested included two strains of *S. arboricolus* [28, 29, 41].

For vegetative growth, yeast extract peptone dextrose (YPD) medium [1 % (w/v) yeast extract (Oxoid); 2 % (w/v) Bacto-Peptone (Oxoid); 2 % (w/v) glucose] was used. Cultures were cryopreserved in 20 % glycerol at −80 °C. Most strains can be obtained from the National Collection of Yeast Cultures (NCYC; see http://www.ncyc.co.uk/ for information). All isolates were stored at −80 °C in a 96-well plate format in 20 % glycerol. More detailed information on each strain and species can be found in the supplementary file (S1).

Phenotypic microarray analysis

For phenotypic microarray (PM) analysis using Biolog, the 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 (NS × 48—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). 3 and 4 % (v/v) 1-butanol was used to induce butanol stress. The final volume was made up to 30 μL using sterile distilled water and dispensed to individual microtitre plate wells. Strains were prepared for inoculation onto PM assay plates as follows. Glycerol stocks stored at −80 °C were streaked on to YPD plates to obtain single colonies and incubated at 30 °C for approximately 48 h. Two to three colonies from each strain were then patched on 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^{-1}) with sterile distilled water using a turbidometer. Cell suspensions for the inoculi were then prepared by mixing 125 μL of these cells and 2.5 mL of IFY buffer™ (Biolog, USA) and the final volume adjusted to 3 mL using RO sterile distilled water. 90 μL of this mix was inoculated to each well in a Biolog 96-well plate. Anaerobic conditions were generated by placing each plate into a PM gas bag (Biolog, Hayward, CA, USA) and vacuum packed using an Audion VMS43 vacuum chamber (Audion Elektro BV, Netherlands).

The OmniLog reader photographs the plates at 15 min intervals to measure dye conversion. The pixel intensity in each well is then converted to a signal value reflecting cell growth. After completion of the run, the signal data are compiled and exported from the Biolog software and compiled using Microsoft® Excel. In all cases, a minimum of three replicate PM assay runs were conducted, and the mean signal values are presented.

Variation was ranked according to the impact on metabolic output defined here as percentage of redox signal intensity of stressed yeast (3 and 4 % 1-butanol) when compared with control and plotted using data from the 25 h time point (the 25 h time point was chosen, as the majority of curves are at exponential or early stationary phase of metabolic output at this time point).

Spot plating

Cryopreserved yeast cells were propagated overnight in 5 mL YPD (yeast extract 10 g/L, peptone 20 g/L, glucose 20 g/L) at 30 °C. The cultures were centrifuged for 4 min at 17, 000*g* at 4 °C. The pellet was collected and washed with sterile distilled water and re-suspended in 100 μL of sterile water. The turbidity of the cultures was measured at 600 nm and cells were diluted to an initial OD_{600} of 1. Sterile distilled water was added to the cultures to prepare samples with 10^2, 10^3 and 10^4 dilution factor. A 5 μL aliquot from each tenfold dilution was spotted onto YPD agar plates containing 0 or 3 % 1-butanol (v/v) and incubated at 30 °C for 48 h. Observations were undertaken at 24 and 48 h and the plates were prepared in duplication.

Confirmation of phenotypic microarray results using mini-fermentation vessels

Fermentations were conducted using 180 mL fermentation vessels. To prepare cells for pitching, cryopreserved yeast of selected strains were inoculated in 5 mL YPD and incubated for 2 days at 30 °C. After 2 days, yeast cultures were sub-cultured in 100 mL of fresh YPD and incubated in an orbital shaker overnight at 30 °C. This was followed by transferring the cultures into a 500 mL conical flask with fresh YPD and incubating for 2 days at 30 °C. The cultures were centrifuged at 17,000×*g* for 5 min and the supernatant was removed. The cells were re-suspended in 5 mL sterile water.

Under control conditions, 1.5×10^7 cells/mL of each strain were inoculated into 98.5 mL of medium containing 4 % (w/v) glucose, 2 % (w/v) peptone, 1 % (w/v) yeast extract and 1.5 mL sterile distilled water. Under 1-butanol stress, 1.5×10^7 cells/mL of each strain were inoculated into 98.5 mL of medium containing 4 % (w/v) glucose, 2 % (w/v) peptone, 1 % (w/v) yeast extract and 1.5 % (v/v) 1-butanol. The volumes of the media were adjusted to account for the addition of 1-butanol to ensure that all fermentations began with the same glucose load.

Anaerobic conditions were applied using a sealed butyl plug (Fisher, Loughborough, UK) and aluminium caps (Fisher Scientific). A Bunsen valve attached to a hypodermic needle was poked through a rubber septum to assist CO_2 release. Fermentations were conducted in triplicate and weight loss was measured at every time point. All experiments experienced incubation at 30 °C, with orbital shaking at 200 rpm for 48 h.

Hierarchical clustering analysis of fermentation stress conditions

The hierarchical clustering algorithm used is based closely on the average-linkage method of Sokal and Michener [37, 38]. The object of this algorithm is to compute a dendrogram that assembles all elements into a single tree. The matrix is scanned to identify the highest value (representing the most similar pair of strains) in comparison with the

reference yeast strain *S. cerevisiae* S288C. Gene Cluster 3.0 was used to construct matrices [13] which are compatible with TreeView for production of representative dendrograms [13, 34].

Detection of glucose, ethanol and 1-butanol from FV experiments via HPLC

Glucose, ethanol and 1-butanol were quantified by HPLC. The HPLC system included a Jasco AS-2055 Intelligent autosampler (Jasco, Tokyo, Japan) and a Jasco PU-1580 Intelligent pump (Jasco). The chromatographic separation was performed on a Rezex ROA H^+ organic acid column of 5 μm and 7.8 × 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 40 min. All chemicals used were of analytical grade (>95 % purity, Sigma-Aldrich, UK).

Quantitative PCR analysis

Yeast strains (*S. cerevisiae* YPS128 and *S. cerevisiae* UWOPS05-227.2) were grown to mid-logarithmic stage of growth in YPD at 30 °C and stressed by the addition of 1.5 % or 3 % (v/v) 1-butanol for 15 min, rotated at 150 rpm. Cells were broken with glass beads using a MagNalyser (Roche, Burges Hill, UK) bead beater for 30 s at 4 °C before incubating on ice for 15 min to precipitate proteins. Cell debris and proteins were pelleted in a microcentrifuge for 15 min (17,000g at 4 °C). Cell-free supernatant was used for the extraction of RNA using an isolation kit from Qiagen (Hilden, Germany), and cDNA was prepared using a first-strand cDNA synthesis kit (GE Healthcare, Bucks, UK).The qPCR conditions were as follows: 0.5 ng/μL cDNA, 6.25 μM forward primer, 6.25 μM reverse primer, 5 μL 2× SYBR Green Master Mix (Applied Bio Systems) and made up to 20 μL using molecular-grade water. All data were compared against the internal standard *ACT1* and data presented as fold change in comparison to *ACT1* transcript levels in control and stress conditions.

Bioinformatics for protein sequence analysis

Open-reading frame (ORF) regions of *RPN4* were obtained from SGD (*Saccharomyces* Genomic Database) and blasted against SGRP (*Saccharomyces* Genome Resequencing Project) (http://www.sanger.ac.uk/cgi-bin/blast/submitblast/s_cerevisiae_sgrp) to identify these genes in candidate *Saccharomyces* spp. strains. Using TBLASTN SGRP, the corresponding protein sequences of each target protein were obtained. To identify sequence similarity, amino acid sequences were multiply aligned using AlignX® Vector NTI Database (Life Technologies, UK) and any differences in protein sequences indicated that the corresponding genes might be significantly tolerant to butanol in *Saccharomyces* yeast.

Statistical analysis

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

Results

Phenotypic variation and ranking of responses of yeast strains to 1-butanol

The phenotypic response to 1-butanol in terms of the metabolic output (as measured by an increase in redox signal intensity) for strains of *Saccharomyces* spp. was assessed using a 96-well microarray assay. The presence of 3 % 1-butanol reduced the metabolic output when compared with unstressed controls (Fig. 1a, b); however, there was variation in metabolic output between yeast strains (Fig. 1a, b).

Tolerance to 1-butanol was ranked and, using this ranking system, *S. cerevisiae* YPS128, DBVPG1788 and DBVPG6044 were identified as being tolerant to 3 % (v/v) 1-butanol and 4 % 1-butanol when compared with other *Saccharomyces* spp. strains (Supplementary data set-S1), comparing metabolic output in assays containing 1-butanol with control experiments it was observed that these strains exhibited metabolic activity of between 70 and 80 % of control metabolic activity in the presence of 1-butanol (Supplementary Figure S1). *S. uvarum* species (DBVPG6299, L-1764, UWOPS99-807.1.1) were observed to display the highest sensitivity to 3 % (v/v) 1-butanol when compared with other *Saccharomyces* spp. strains (Fig. 2a). These strains exhibited almost no metabolic output in the presence of 3 % 1-butanol when compared with metabolic output under control conditions (Supplementary File S1). *S. cerevisiae* S288C used as a control strain for these experiments was in the bottom 30 in terms of sensitivity to 3 % butanol (Fig. 2a).

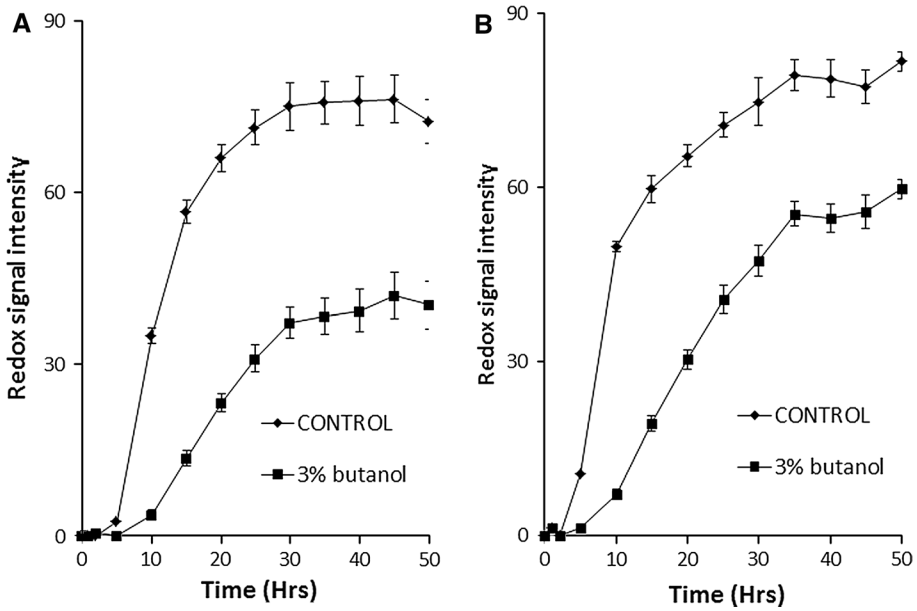

Fig. 1 Phenotypic microarray analysis under control conditions and in the presence of inhibitory compounds. **a** Redox signal intensity under control conditions and in the presence of 3 % 1-butanol for *S. cerevisiae* S288C. **b** Redox signal intensity under control conditions and in the presence of 3 % 1-butanol for *S. cerevisiae* YPS128. Data are representative of triplicate values

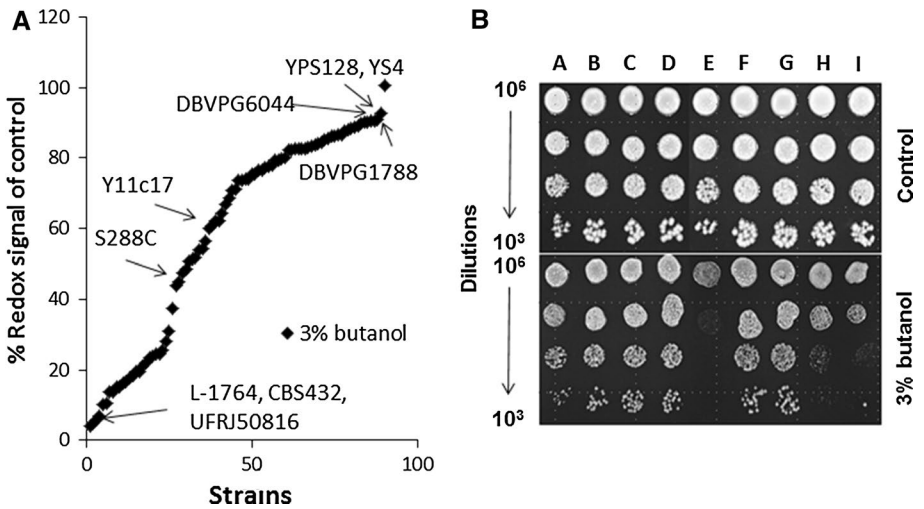

Fig. 2 Phenotypic microarray analysis (redox signal intensity) and assessment of growth for *Saccharomyces* spp. with 1-butanol. **a** 3 % 1-butanol; the values shown are an average of triplicate experiments. **b** Spot test assay in YPD plates in the presence and absence of 1-butanol after 24 h of incubation. Within the figure—A: *S. cerevisiae* S288C; B: *S. cerevisiae* YIIc17_E5; C: *S. cerevisiae* DBVPG1788; D: *S. cerevisiae* YS4; E: *S. paradoxus* CBS432; F: *S. cerevisiae* DBVPG6044; G: *S. cerevisiae* YPS128; H: *S. paradoxus* UFRJ50816; I: *S. uvarum* L-1764

Effect of 1-butanol on the growth of *Saccharomyces* spp.

Spot tests were also carried out to determine the viability of *Saccharomyces* spp. strains in the presence of 3 % (v/v) 1-butanol. Selected tolerant strains (*S. cerevisiae* YPS128, YS4, DBVPG1788 and DBVPG6044) displayed tolerance to 1-butanol when compared with a reference yeast strain *S. cerevisiae* S288C and selected sensitive strains (*S. uvarum* L-1764, *S. paradoxus* CBS432 and UFRJ50816) (Fig. 2b). Growth for all *Saccharomyces* spp. yeasts was inhibited on plates containing more than 3 % (v/v) 1-butanol (data not shown).

Confirmation of phenotypic microarray strain assessments using mini-fermentation analysis

The objective of this study was to identify potential candidate strains for efficient second-generation bio-butanol fermentations. Fermentation progression was monitored by measuring glucose utilisation/ethanol production in the presence of 1-butanol when compared with unstressed controls. Ethanol production was used as a parameter for yeast performance under 1-butanol stress as pyruvate a key component in 1-butanol synthesis can also be converted

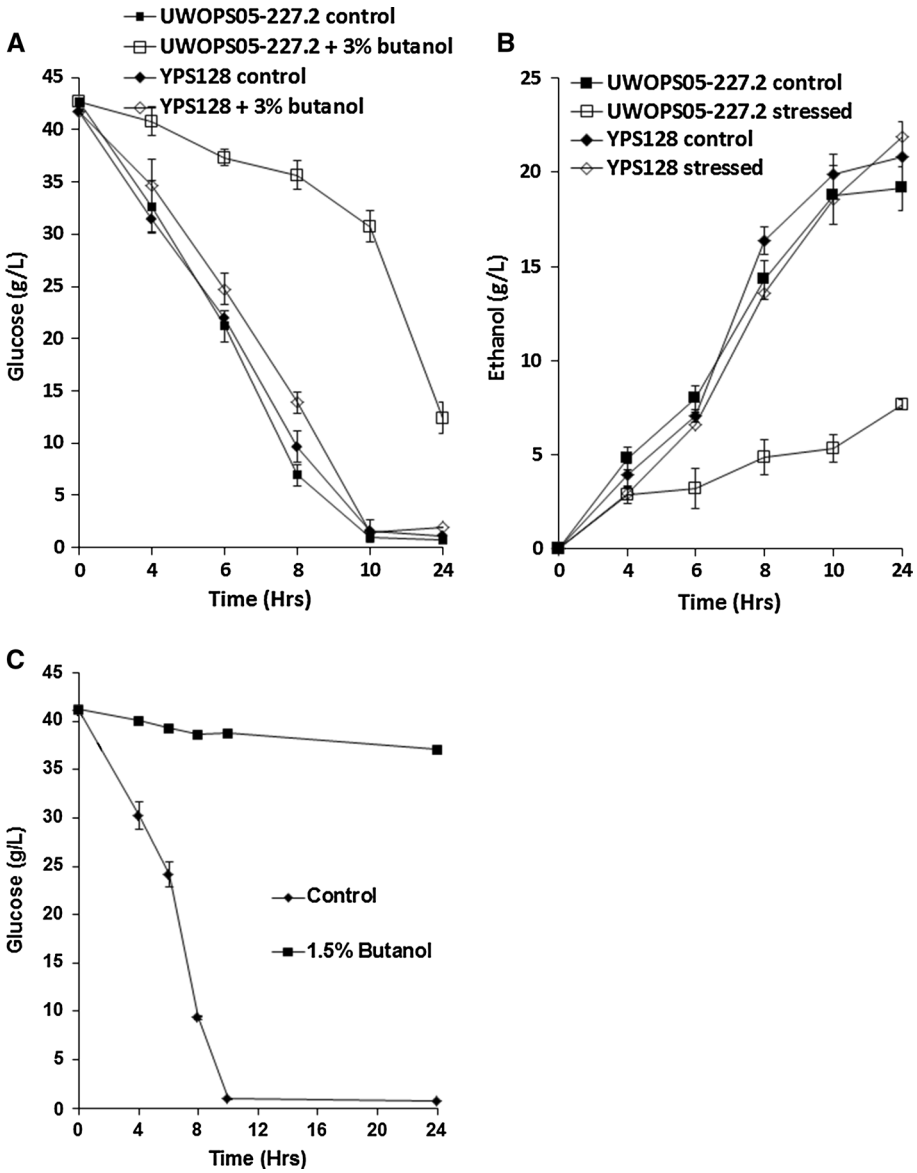

Fig. 3 A comparison between *S. cerevisiae* UWOPS05-227.2, *S. uvarum* L-1764 and *S. cerevisiae* YPS128 strains under control conditions and in the presence of 1.5 % (v/v) 1-butanol. **a** Glucose utilisation (g/L) for *S. cerevisiae* YPS128 and *S. cerevisiae* UWOPS05-227.2, **b** ethanol production (g/L) for *S. cerevisiae* YPS128 and *S. cerevisiae* UWOPS05-227.2, **c** glucose utilisation (g/L) for *S. uvarum*. Each data point represents the mean value of biological triplicate experiments with SD *error bars*

into ethanol [22]. After screening via phenotypic microarrays for tolerance to 1-butanol, glucose utilisation and ethanol production for tolerant *S. cerevisiae* strain YPS128 and 1-butanol-sensitive *S. cerevisiae* UWOPS05-227.2 under control or in the presence of 1-butanol was determined. All strains had reduced rates of glucose utilisation and ethanol production in the presence of 1-butanol when compared with non-stressed controls. After 8 h of fermentations, there was 7 and 9 g/L glucose remaining in the fermentation under control conditions for *S. cerevisiae* YPS128 and *S. cerevisiae* UWOPS05-227.2, respectively, compared with 14 and 35 g/L for the same strains under 3 % 1-butanol stress (Fig. 3a, b). Glucose was utilised in fermentations using *S. cerevisiae* YPS128 after 10 h; however, *S. cerevisiae* UWOPS05-227.2 still had residual glucose in the fermentation after 24 h in the presence of 1-butanol (Fig. 3a). Glucose utilisation and ethanol production under control conditions was almost identical for the *S. cerevisiae* strains (Fig. 3a, b). Fermentations with *S. uvarum* (L-1764) under 1.5 % 1-butanol stress was characterised by a lack of glucose utilisation (Fig. 3c) and no ethanol production (data not shown). The concentrations of 1-butanol were determined during the fermentations and the levels remained unchanged throughout the fermentations (data not shown).

RPN4 was significantly up-regulated in 1-butanol-tolerant yeast

A 1-butanol-tolerant *S. cerevisiae* yeast strain (YPS128) and a sensitive yeast strain (UWOPS05-227.2) were stressed with the addition of 1.5 % (v/v) or 3 % (v/v) 1-butanol and the selected genes were analysed for changes

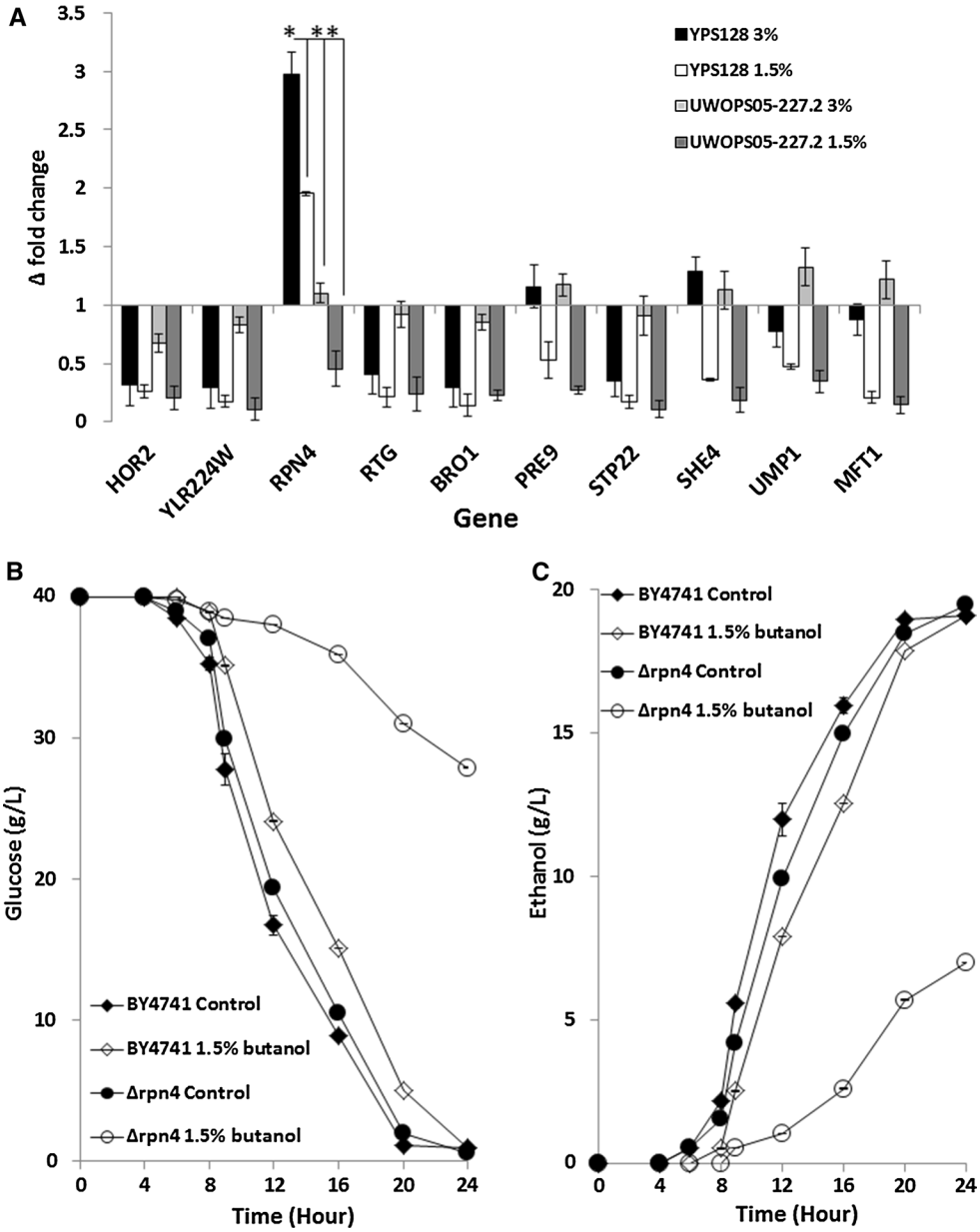

Fig. 4 Expression data (Δ fold change) from *S. cerevisiae* YPS128 and *S. cerevisiae* UWOPS05-227.2 for selected genes. **a** Under 1.5 % (v/v) and 3 % 1-butanol stress when compared with unstressed controls. **b** Glucose utilisation (g/L) during fermentation for BY4741 and Δ*rpn4* under control and in the presence of 1.5 % (v/v) 1-butanol. **c** Ethanol production (g/L) during fermentation for BY4741 and Δ*rpn4* under control and in the presence of 1.5 % (v/v) 1-butanol. Each data point represents the mean value of biological triplicate experiments with SD *error bars*

in expression. The selection of the genes was determined using previously published work, in which genes have been highlighted as being up-regulated under 1-butanol and ethanol stress [17].

The majority of the genes after exposure to 1.5 % (v/v) 1-butanol were down-regulated in both tolerant and sensitive yeast strains; however, *RPN4* was significantly up-regulated in the 1-butanol-tolerant *S. cerevisiae* YPS128 (Fig. 4a). Similarly, *RPN4* exhibited significant up-regulation in the same yeast when under 3 % (v/v) 1-butanol stress; however, this gene was not up-regulated in the 1-butanol-sensitive yeast (UWOPS05-227.2) after exposure to 1.5 % (v/v). However, we detected a slight up-regulation of *RPN4* in the presence of 3 % (v/v) 1-butanol, though this was significantly reduced compared with up-regulation in YPS128 (Fig. 4a). We also observed an up-regulation of *RPN4* in *S. cerevisiae* DVBPG6044 in the presence of 1-butanol when compared with unstressed conditions (data not shown).

On comparing peptide sequences for RPN4 from 1-butanol-tolerant and sensitive *S. cerevisiae* strains, it was observed that there was conserved alteration in the peptide sequence between tolerant and sensitive strains; however, two 1-butanol-sensitive strains have a histidine at residue 444 rather than a leucine present in the majority of *S. cerevisiae* strains (Supplementary Figure S2). The importance of this residue in terms of tolerance to 1-butanol is currently being investigated.

To determine the importance of *RPN4* in 1-butanol-stressed yeast cells we examined how a Δ*rpn4* knockout behaved in the presence of 1-butanol. Fermentations were carried out under control and in the presence of 1-butanol using a haploid reference strain (BY4741). *S. cerevisiae* BY4741 was used for these experiments rather than a wild-type polyploidy *Saccharomyces* strain. BY4741 exhibited identical 1-butanol tolerance to S288C, which is highlighted in Fig. 2a, and a Δ*rpn4*-deletion strain with glucose utilisation and ethanol production measured at specific time points throughout the experiment. The presence of 1-butanol reduced the rates of glucose utilisation and ethanol production for BY4741 and Δ*rpn4* when compared with unstressed controls (Fig. 4b, c); however, BY4741 in terms of glucose utilisation and ethanol production was significantly more tolerant to 1-butanol when compared with Δ*rpn4* (Fig. 4b, c). *RPN4* was not up-regulated in BY4741 cells in the presence of 1.5 or 3 % 1-butanol (data not shown).

Discussion

Utilising a phenotypic microarray analysis, *Saccharomyces* spp. strains were screened for tolerance to the presence of 1-butanol and there was significant variation in response.

Phenotypic microarrays have been used previously to screen yeast for phenotypes such as tolerance to acetic acid or ethanol [43]. When yeasts were subjected to 1-butanol stress, we observed considerable variation in response with *S. cerevisiae* YPS128 and DBVPG6044 being most tolerant and *S. uvarum* (L-1764) the most sensitive. *S. uvarum* strains have previously been shown to be sensitive to the presence of inhibitory compounds [43], whilst many of the *S. cerevisiae* strains involved in this study have been reported to be inherently ethanol tolerant [4], and ethanol-tolerant yeast strains have been used to improve tolerance to 2-butanol using sequential re-pitching [16]. *S. cerevisiae* YPS128 was not inhibited by the presence of 1-butanol when compared with control conditions under fermentation conditions. Comparing 1-butanol tolerance with tolerance to ethanol published previously [43], we failed to observe any correlation between tolerance to 1-butanol and ethanol.

It has previously been reported that environmental stresses, including exposure to butanol, lead to changes at the molecular level of the yeast cell, leading to alterations in gene expression and resulting in reduced growth rate [15]. Several genes have been highlighted as being relevant to yeast cells for tolerating 1-butanol stress [16, 17]. Examining the expression of these candidate genes in 1-butanol-tolerant or -sensitive *S. cerevisiae* strains, it was observed that the majority were down-regulated in the presence of 1-butanol, including *HOR2*. The overexpression of *HOR2* has been linked to a butanol-tolerant phenotype [16]. *RPN4* was significantly up-regulated in the presence of 1-butanol in the 1-butanol-tolerant yeast *S. cerevisiae* YPS128 when compared with the 1-butanol-sensitive *S. cerevisiae* UWOPS05-227.2.

Previously published work has demonstrated that mutated alleles of *RPN4* have been identified from 1-butanol-tolerant yeast haploids and it has been suggested that mutated alleles may participate in protecting the yeast cell against 1-butanol stress [17]. The same research also highlighted *RTG* as a gene in which allelic variation was observed between a yeast strain with improved butanol tolerance compared with the parental strain [17]; however, we failed to observe an up-regulation of *RTG* between different yeast strains in this study. Two 1-butanol-sensitive strains carry a histidine at residue 444 rather than a leucine which is more common in RPN4 peptides in yeast strains. Mutations in the RPN4 protein have been shown to improve yeast tolerance to butanol [17]. Though none of the strains contained the mutation described previously, other mutations in the RPN4 correlating with butanol tolerance have not been identified. A systematic site-directed approach within the RPN4 protein correlating with butanol tolerance would be a worthwhile experiment.

When we assessed Δ*rpn4* null mutants for tolerance to 1-butanol during fermentation, it was observed that Δ*rpn4* was sensitive to the presence of 1-butanol when compared with controls. RPN4 is a proteasome protein with a very short half-life [44], promoting the expression of proteasome genes as well as working as a negative feedback on its own expression [42], The influence of the proteasome on protein folding has been suggested as a reason for butanol tolerance [17]. Proteasome activity has been associated with cells under oxidative stress. The 20S proteasomal subunit has been shown to be subjected to *S*-glutathionylation as an adaptive response [11], and cells accumulate reactive oxygen species when under constant exposure to ethanol or butanol [35]. Work is progressing on studying whether the overexpression of *RPN4* has an influence on redox homoeostasis, proteasome activity and 1-butanol tolerance.

Our approach has highlighted that screening *Saccharomyces* spp. for response to butanol can identify strains with inherent tolerance to 1-butanol. These strains would be a useful starting point for further strain development for the production of 1-butanol from hydrolysates derived from lignocellulosic material in yeast fermentation.

Conclusion

The study was set out to explore the opportunities for improvement in biofuel commercial production through identification of 1-butanol-tolerant strains and associate

genes facilitating the increased tolerance to 1-butanol stress. Screening of *Saccharomyces* spp. yeasts and analysis of tolerant and sensitive strains under 1-butanol stress revealed that a transcription regulator of proteasome genes, *RPN4*, was up-regulated in tolerant yeast strains when compared with sensitive strains.

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Conflict of interest We declare that the authors have no conflicts of interest in connection with this paper.

Author's contributions AMZ performed the experiments, TTW and DG designed the experiments and DG wrote the paper.

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