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RESEARCH ARTICLE

Physiological responses to acid stress by *Saccharomyces cerevisiae* when applying high initial cell density

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One sentence summary: The physiological response of yeast to acetic, formic, levulinic and cinnamic acids was studied during aerobic cultivation of *S. cerevisiae* at high initial cell density.
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ABSTRACT

High initial cell density is used to increase volumetric productivity and shorten production time in lignocellulosic hydrolysate fermentation. Comparison of physiological parameters in high initial cell density cultivation of *Saccharomyces cerevisiae* in the presence of acetic, formic, levulinic and cinnamic acids demonstrated general and acid-specific responses of cells. All the acids studied impaired growth and inhibited glycolytic flux, and caused oxidative stress and accumulation of trehalose. However, trehalose may play a role other than protecting yeast cells from acid-induced oxidative stress. Unlike the other acids, cinnamic acid did not cause depletion of cellular ATP, but abolished the growth of yeast on ethanol. Compared with low initial cell density, increasing initial cell density reduced the lag phase and improved the bioconversion yield of cinnamic acid during acid adaptation. In addition, yeast cells were able to grow at elevated concentrations of acid, probable due to the increase in phenotypic cell-to-cell heterogeneity in large inoculum size. Furthermore, the specific growth rate and the specific rates of glucose consumption and metabolite production were significantly lower than at low initial cell density, which was a result of the accumulation of a large fraction of cells that persisted in a viable but non-proliferating state.

Keywords: weak acids; *Saccharomyces cerevisiae*; physiological response; high initial cell density; low initial cell density

INTRODUCTION

Weak acids, such as acetic, propionic, benzoic and sorbic acids, are commonly used as preservatives to limit unwanted microbial growth in the food and beverage industry (Teixeira, Mira and Sá-Correia 2011). The inhibitory effect imposed by weak acids on microorganisms has partially been ascribed to the 'uncoupling' mechanism (Russel 1992). Accordingly, at low pH, weak acids prevail in undissociated form, being more hydrophobic and prone to crossing the membrane by simple passive diffusion. Once inside the cell, weak acids will dissociate in the near-neutral cytosol and release protons, which can potentially acidify the cell and cause dissipation of the proton-motive force (Kotyk and Georghiou 1991; Imai and Ohno 1995; Guldfeldt and Arneborg 1998). In addition, the anion of the weak acid accumulates in the cell and may reach a toxic level and affect the metabolic functions of the cell (Stratford and Anslow 1998;

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Bauer *et al.* 2003). The antimicrobial effects of weak acids are determined by the chemical properties of the acid, in particular by the hydrophobicity and pK_a of the acid (Mira, Teixeira and Sá-Correia 2010).

The resistance mechanisms that microorganisms develop to counteract the impact of weak acids have been intensively studied (as reviewed in Brul, Kallemeijn and Smits 2008; Mira and Teixeira 2013). It has been demonstrated that acid-challenged *Saccharomyces cerevisiae* maintained intracellular pH homeostasis by ATP-dependent efflux of the protons via plasma membrane H^+-ATPase Pma1p (Carmelo, Santos and Sá-Correia 1997). Extrusion of anions accumulated in the cell has been reported to take place by ATP-binding cassette (ABC) transporter through an ATP-driven process in *S. cerevisiae* (Holyoak *et al.* 1999; Hatzixanthis *et al.* 2003). However, the actions of H^+-ATPase and Pdr12 (an ATP-binding cassette transporter) in response to acid stress result in significant increase in energy (ATP) demand, thereby leaving less ATP for cell growth (Holyoak *et al.* 1996).

In lignocellulosic hydrolysate fermentations, inhibitory compounds such as furan derivatives, weak acids and phenolics often hamper the fermentation ability of *S. cerevisiae* (Palmqvist and Hahn-Hägerdal 2000a). In order to overcome the impact of inhibitors on yeast metabolism, high initial cell density has often been employed to enhance the detoxification of some inhibitors and achieve a high volumetric fermentation rate (Chung and Lee 1984). For instance, enhanced rates of converting furfural and hydroxymethylfurfural to less inhibitory alcohol forms have been observed in *S. cerevisiae* by using high initial cell density (Chung and Lee 1984). In addition, detoxification of some phenolic acids is also expected to be enhanced at high initial cell density of *S. cerevisiae* as this yeast harbors phenylacrylic acid decarboxylase (PAD1), which can convert cinnamic, *p*-coumaric and ferulic acids to the corresponding vinyl derivatives (Larsson, Nilvebrant and Jönsson 2001). It has been reported that the need to elevate pH to alleviate the toxicity of weak acid decreased when a high initial cell density was applied (Palmqvist and Hahn-Hägerdal 2000a). However, in order to better understand the possible effects of using high initial cell density to decrease the effect of weak acid, a detailed physiological study on yeast *S. cerevisiae* at high initial cell density cultivation in the presence of common weak acids that can be found in lignocellulosic hydrolysate is called for.

In our previous study, we investigated the physiological response of *S. cerevisiae* during aerobic culture, starting from a low cell density (0.1 g dry cell weight (DCW) l^{-1}) under acid stress (Guo and Olsson 2014). We found that the growth of yeast cells on glucose was impaired by the acids. Acids at moderate concentrations can stimulate glycolytic flux, while higher levels of acid slow down the glycolytic flux. Yeast cells under acid stress showed similar energy levels compared with the non-stressed cells in the exponential growth phase. Moreover, slightly higher cellular trehalose content was observed in acid-stressed cells compared with reference cultures.

This study examines *S. cerevisiae* physiology under acetic, formic, levulinic and cinnamic acid stress in aerobic batch cultures of high initial cell density. Aerobic cultivation conditions were chosen as aerobic production of chemicals and proteins has been commonly used for engineered *S. cerevisiae*, while they have been less studied than anaerobic cultivation conditions (Abbott *et al.* 2009). Among the acids studied here, formic acid has a lower pK_a value (3.75 at 20°C) than acetic acid (pK_a 4.79 at 20°C) and levulinic acid (pK_a 4.66 at 20°C). Cinnamic acid, a phenolic acid commonly found in hydrolysates (Almeida *et al.* 2007), is considered to exhibit high anion toxicity due to its high hydrophobicity (reflected in a log partition octanol/water coefficient of 2.1) (Brul, Kallemeijn and Smits 2008). The following hypotheses were addressed: (i) regardless of initial cell density a specific acid will cause similar physiological responses of yeast cells, (ii) each acid will induce general and acid-specific responses of *S. cerevisiae*, and (iii) the increase in phenotypic cell-to-cell heterogeneity in a large inoculum size will improve the tolerance of *S. cerevisiae* to weak acid stress in high initial cell density cultivations.

MATERIALS AND METHODS

Yeast strain and media

The haploid, prototrophic *Saccharomyces cerevisiae* strain CEN.PK 113–7D (*MATa*) was used in this study and stored in 50% glycerol at −80°C. Yeast cells taken from frozen stocks were grown on YPD medium containing 10 g l^{-1} yeast extract, 20 g l^{-1} peptone and 20 g l^{-1} glucose. All the experiments for studying physiological response of yeast to acid stress were performed in defined medium according to Verduyn *et al.* (1992) containing 25 g l^{-1} glucose and acids at the indicated concentrations.

Growth conditions

Cultivation of yeast cells was routinely carried out at 30°C. The inoculum culture was inoculated with one single colony of yeast from a YPD agar plate that was transferred to 100 ml of defined medium (as described above) and cultivated until exponential growth phase in a 500 ml Erlenmeyer flask, when the cells were harvested by centrifugation at 6000×*g* for 5 min. The cell pellet was washed, resuspended in an appropriate volume of defined medium, and then transferred into bioreactors to yield an initial biomass concentration of 1.0 g DCW l^{-1}, referred to as high initial cell density (Moniruzzaman *et al.* 1997). Batch cultivation was conducted in a 3.0 l DASGIP bioreactor (DASGIP BioTools, LLC, USA) with a working volume of 2.0 l. The pH was controlled at 5.0 with the automatic addition of 2 M KOH. An aeration of 0.5 vvm was used and the stirring speed was set to 600 rpm to ensure a dissolved oxygen tension of at least 60% of air saturation. Acetic, formic, levulinic or cinnamic acid was added to the medium. Exhaust gas from the fermenter cultures was cooled in a condenser (2°C) and dried. Oxygen and carbon dioxide concentrations were determined with a DASGIP Off-Gas Analyzer GA4. To prevent excessive foaming, 0.15 ml l^{-1} silicone antifoam (Sigma-Aldrich) was added. Samples were taken regularly for later determination of biomass concentration, extracellular metabolites including glucose, ethanol, glycerol, acetate, pyruvate and succinate, intracellular oxidation level, intracellular nucleotides (ATP, ADP and AMP), and the content of trehalose and glycogen. The results are shown as the mean value of three independent experiments.

Dry weight determination

Culture samples (two of 10 ml each) were filtered over pre-weighted PES filters (0.45 μm; Sartorius Biolab, Germany). The retained biomass on filters was washed, dried in a microwave oven at 150 W for 15 min, and then stored in a desiccator before being weighed.

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Determination of substrate and extracellular metabolites

Glucose, glycerol, ethanol, acetate, pyruvate, succinate, formic acid and levulinic acid of the culture broth were analyzed using an Aminex HPX87-H column (Bio-Rad Laboratories, Germany), with 5 mM H_2SO_4 as the mobile phase at a flow rate of 0.6 ml min^{-1}. The column was operated at 60°C. Glucose, glycerol, ethanol and acetic acid were detected with a refractive index detector (Shodex RI-101; Showa Denko, New York, NY, USA). Pyruvate, succinate, formic acid and levulinic acid were detected using an UV detector at 210 nm (Dionex, Sunnyvale, CA, USA). Cinnamic acid and potential degradation products were measured by gas chromatography–mass spectrometry as described previously (Larsson, Nilvebrant and Jönsson 2001).

Calculation of specific rates of substrate consumption and product yield in exponentially growing batch cultures

Samples were taken at appropriate intervals during exponential growth to determine the dry weight of yeast cultures, substrate and product concentrations in the supernatant. Values for specific rate of glucose consumption, q_{glc} (mmol g^{-1} h^{-1}), were calculated using the following equation: $q_{glc} = \mu_{max}/Y_{X/S}$, where $Y_{X/S} = dX/dS$ (biomass yield coefficient) was calculated using linear regression of biomass concentration (X) *vs* substrate concentration (S) during the exponential growth phase. The maximum specific growth rate on glucose (μ_{max}; h^{-1}) was estimated from the exponential part of a plot of biomass concentration *vs* time. Similarly, to calculate the specific production rate of ethanol, glycerol, acetate and pyruvate ($q_{ethanol}$, $q_{glycerol}$, $q_{acetate}$, $q_{pyruvate}$ and $q_{succinate}$), $Y_{X/S}$ was replaced by the yield of each corresponding metabolite. Specific rates of CO_2 production and O_2 consumption during exponential growth were calculated as described previously (van Urk *et al.* 1998). The calculations of flux rates and μ_{max} were based on the viable cell fractions of total cell population as determined by the plate-counting method, and total biomass concentration, respectively. Lag phase was estimated by using DMFit (www.ifr.ac.uk/safety/DMfit) as described previously (Rolfe *et al.* 2012). The product yields of metabolites including ethanol, glycerol, acetate, pyruvate and succinate were obtained as the slope of the linear curve when the metabolite concentration was plotted against the glucose concentration during exponential growth on glucose, unless stated otherwise. Biomass yield was calculated as the final biomass produced divided by the amount of substrate consumed.

Ethanol evaporation rate changes with the change in volume of the medium in bioreactors due to sampling. To correct for this, ethanol evaporation kinetics were analyzed in bioreactors operated under identical conditions at different working volumes with cell-free medium. The resulting volume-dependent ethanol evaporation constants (0.016 divided by the volume in liters, expressed in h^{-1}) were used to correct ethanol concentrations. All data are presented as mean values of at least three biological replicates ± standard deviation.

Determination of viability

Viable-cell counts were determined by the standard plate-counting method. Briefly, culture samples were serially diluted (dilution factors 10^{-1}–10^{-4}) in sterile distilled water and 200 μl of each dilution was spread on solid YPD medium. Plates were incubated at 30°C for 2 days and colony forming units (CFU) in dilutions resulting in the range of 50–150 colonies per plate were included for counting. To determine the total cell population, cultures were diluted in sterile distilled water to reach a density of approximately 1.0×10^7 cells ml^{-1} and examined under the microscope (Thomas and Ingledew 1990). Viability was indicated as the percentage of viable cells in the total cell population. The measurements of viable cell and total cell numbers were performed for at least five biological replicates to reduce the errors of these methods in the determination of cell numbers in general.

Assessment of yeast vitality using acidification power test

Yeast vitality, referring to the yeast activity in sugar utilization, was indicated by glucose-induced proton efflux (GIPE) and determined by the acidification power (AP) test as described previously (Siddique and Smart 2000). The AP test contains two calculated components, water acidification power (WAP), which measures spontaneous H^+ efflux sustained solely by endogenous H^+ and energy sources, and glucose acidification power (GAP), which measures H^+ efflux closely associated with the utilization of both endogenous and exogenous sources. Quantitative measurement of both processes can thus provide information on the level, availability and utilizability of endogenous energy sources and on the ability of the cells to utilize exogenous substrates. The GIPE value deduced from this assay is an indicator of the overall metabolic state of the cells (Opekarová and Sigler 1982).

Briefly, yeast cells were harvested, washed and resuspended in distilled water to reach a final cell concentration of approximate 5.0×10^8 cells ml^{-1}. For WAP, pH was recorded initially (WAP0) after adding 1 ml of cell suspension to 19 ml distilled water. After 10 min, 5 ml distilled water was added to the cell suspension and pH was measured twice at 10 min interval (WAP10 and WAP20). WAP was calculated according to the following equation: WAP = WAP0 – WAP20. For GAP, a similar procedure was used except that instead of distilled water, 5 ml of 20% (w/v) glucose solution was added. The pH was recorded after addition of cells (GAP0), 10 min (GAP10) and 20 min (GAP20) after the addition of glucose solution. WAP was calculated according to the equation: GAP = GAP0 – GAP20. Consequently, GIPE was calculated according to the equation: GIPE = GAP – WAP.

Determination of cellular trehalose and glycogen content

Samples (two with a volume of 10 ml each) were quickly taken from the cultures and immediately placed in liquid nitrogen. Yeast cells were harvested by centrifugation of the samples at 8000 × g for 5 min at 4°C and the cellular trehalose and glycogen contents were measured as described previously (Parrou and François 1997), except that 0.1 U ml^{-1} trehalase (Megazyme Int., Bray, Ireland) was used. In this method, trehalose and glycogen were extracted from yeast by boiling the cells in 0.25 M Na_2CO_3, and then hydrolyzed to glucose by purified trehalase and amyloglucosidase, respectively. The released glucose was quantified with an enzymatic kit (Biosis, Biotechnological Applications Ltd, Athens, Greece).

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Table 1. Effect of weak acids on the length of the lag phase and the maximum apparent specific growth rate at pH 5 under aerobic conditions. The results were calculated from at least three biological replicates, and are given as the mean values ± standard deviation. N/A: not available.

Parameter	Reference (no stress)	Acetic acid (300 mM)	Formic acid (220 mM)	Levulinic acid (400 mM)	Cinnamic acid (0.8 mM)
pK_a	N/A	4.79	3.75	4.66	4.44
log P[a]	N/A	−0.17	−0.54	−0.49	2.13
Undissociated acid (mM)	0	114.4	12.2	121.6	0.17
Lag phase Glu (h)	0	26	4	44	0
$\mu_{max\text{-}glu}$ (h^{-1})	0.26 ± 0.00	0.07 ± 0.01	0.11 ± 0.01	0.09 ± 0.01	0.04 ± 0.01
$Y_{X/S}$ (g DCW·g^{-1} glu)	0.12 ± 0.01	0.06 ± 0.01	0.07 ± 0.01	0.06 ± 0.01	0.05 ± 0.01
Lag phase Eth (h)	0	60	40	75	N/A
$\mu_{max\text{-}eth}$ (h^{-1})	0.10 ± 0.01	0.03 ± 0.01[b]	0.06 ± 0.00	0.01 ± 0.00	N/A

[a]The lipophilic tendency given by the partition coefficient octanol–water (P).
[b]Growth with consumption of ethanol and acetic acid.

Determination of intracellular energy level

Cellular contents of adenosine nucleotides were determined as described previously (Ask *et al.* 2013). Briefly, samples (two of 5 ml each) were quenched in 25 ml pure methanol at −40°C. Cellular ATP, ADP and AMP were extracted by incubation of cell pellets with 0.5 ml 0.52 M trichloroacetic acid containing 17 mM EDTA on ice for 15 min. Adenosine nucleotides were separated on a Luna® 5 μm C18(2) 100 Å LC column (150 × 4.6 mm) (Phenomenex Inc., Torrance, CA, USA) using acetonitrile and tetrabutylammonium buffer (0.005 M tetrabutylammonium hydrogensulfate, 0.01 M Na_2HPO_4, pH 7.0) as mobile phase at a flow rate of 1.0 ml min^{-1} at 20°C. The detection was performed with a photodiode array detector (PDA-3000; Dionex Corp.) at 260 nm. The energy charge was calculated according to the following equation: Energy charge = $(ATP + \frac{1}{2}ADP)/(ATP + ADP + AMP)$

Analysis of intracellular oxidation level

Intracellular oxidation level of yeast was estimated by using the oxidant-sensitive probe 2′,7′-dichlorofluorescin diacetate as described by Nomura and Takagi (2004). The cell pellet was disrupted in Tris–HCl buffer (50 mM, pH 7.4, 3 mM EDTA and 0.5 mM phenylmethylsulfonyl fluoride) using an MP FastPrep-24 Instrument (MP Biomedicals Inc.). The homogenate was centrifuged (10 000×*g* for 10 min) and the cell-free extract was obtained and used for the following fluorescence measurement. Fluorescence was measured with λ_{EX} = 490 nm and λ_{EM} = 524 nm using the Safire II spectrofluorometer (Tecan, Austria). Intracellular oxidation level was defined as the value of λ_{EX} = 524 nm divided by the protein concentration in the cell-free extract. The protein content was determined by a modified Biuret method, using bovine serum albumin as a standard (Verduyn *et al.* 1991).

RESULTS AND DISCUSSION

To investigate the effects of different acids on aerobic *S. cerevisiae* cultures at comparatively high initial cell density (1.0 g DCW l^{-1}), yeast was grown on defined medium containing 25 g l^{-1} glucose at pH 5.0. Acetic, formic, levulinic or cinnamic acid was added to the medium. The physiological stress responses of *S. cerevisiae* to these acids were studied. Yeast cells grown without acid addition were used for comparison and will in the following be referred to as the reference.

Increasing the initial cell density increases the level of acid that *S. cerevisiae* can tolerate

The concentration of each acid required to reduce the biomass yield during growth on glucose by 50% as compared with the reference was determined (Table 1). The undissociated acid (concentrations also shown in Table 1) that can cross the cell membrane by passive diffusion and the anion of weak acids have been shown to be key factors in acid toxicity (Abbott *et al.* 2007; Stratford *et al.* 2013; Ullah *et al.* 2013). It was noticed that the amount of acid required to reduce the biomass yield by half was related to the hydrophobicity of acetic acid, levulinic acid and cinnamic acid. For the most hydrophobic acid, cinnamic acid, the undissociated form at 0.17 mM led to a 50% reduction in biomass yield. Although formic acid is less hydrophobic than acetic and levulinic acids, the amount of undissociated formic acid (12.2 mM) required to decrease the biomass yield by 50% was much lower than that of acetic acid (114 mM) and levulinic acid (106 mM). This implied that a unique chemical property of formic acid is the determinant of its toxicity, which has been ascribed to its small molecular size (Palmqvist and Hahn-Hägerdal 2000b). According to our previous results, a lower amount of undissociated acetic acid (83.9 mM), formic acid (10 mM) and levulinic acid (76.0 mM) was required to reduce biomass yield by half when low initial cell density was used (Guo and Olsson 2014), compared with the high cell densities applied in the current study. In addition, yeast failed to grow in the presence of 300 mM acetic acid when the culture started with an initial cell density of 0.1 g DCW l^{-1}. Therefore, the acid level in the culture that *S. cerevisiae* can tolerate increased by increasing the initial cell density.

Effect of increasing initial cell density on lag phase of *S. cerevisiae* CEN.PK

Saccharomyces cerevisiae exhibited quite different growth profiles with the imposed acid stress compared with the reference condition (Fig. 1). Under the reference conditions, the yeast first grew on glucose and produced ethanol, and then grew on ethanol when glucose was depleted. No obvious lag phase was observed between the two growth phases (Table 1, Fig. 1a). Yeast cells exposed to acetic acid or levulinic acid showed a 26 h and a 44 h lag phase on glucose, respectively, whereas addition of formic acid to the culture only resulted in a 4 h lag phase (Fig. 1b–d). No obvious lag phase was observed for cinnamic acid-stressed cells grown on glucose (Fig. 1e). Comparing with the cultivation process started at a low initial cell density, a lower

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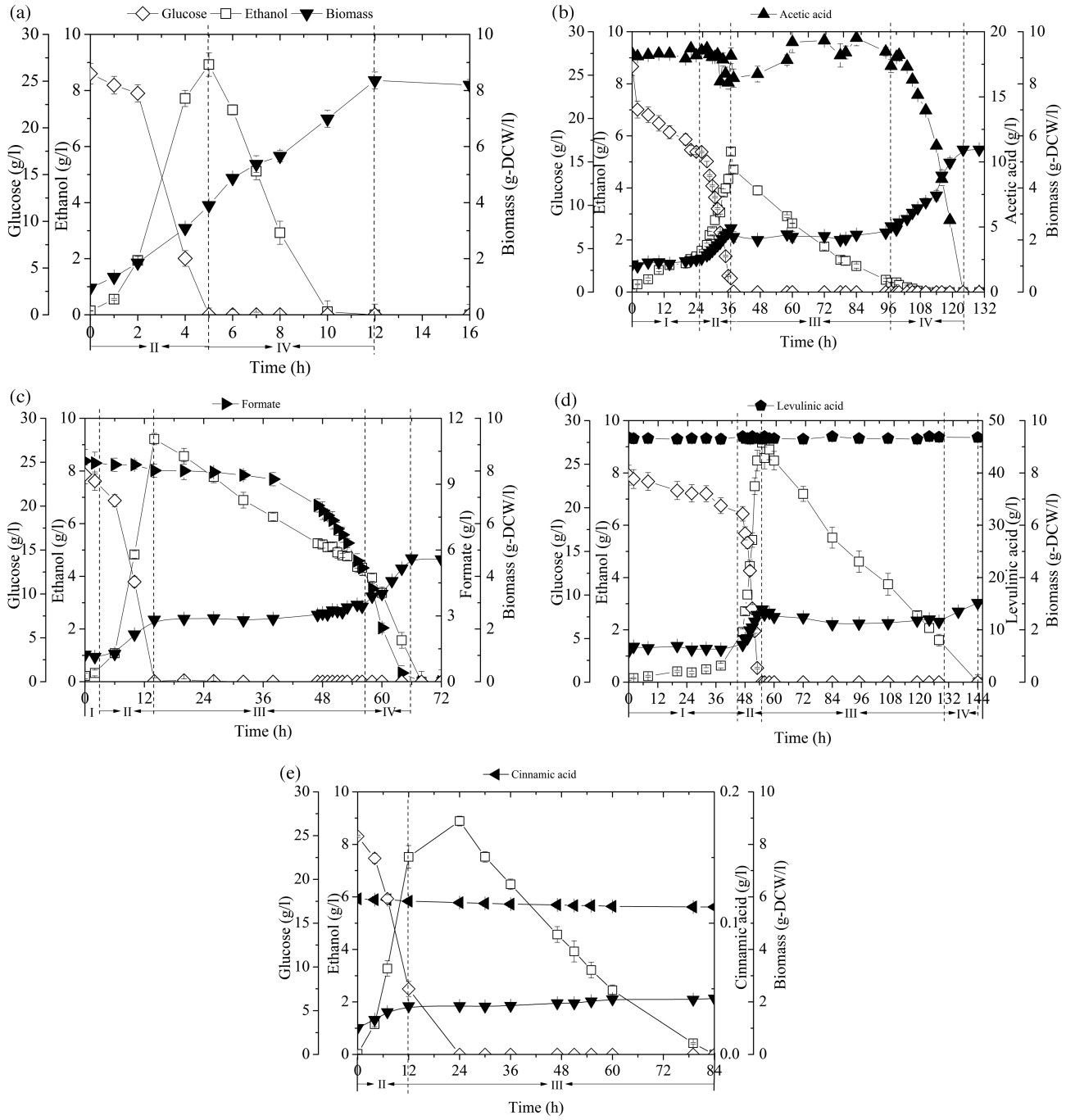

Figure 1. Comparison of ethanol, biomass production and sugar consumption of *S. cerevisiae* during aerobic culture with no acid added (**a**) or with addition of 300 mM acetic acid (**b**), 220 mM formic acid (**c**), 400 mM levulinic acid (**d**) and 0.8 mM cinnamic acid (**e**) at pH 5.0. The cultivation process in the presence of acetic acid, formic acid and levulinic acid was divided by the dashed line into four phases: I, lag phase (adaptation phase); II, growth on glucose; III, lag phase; and IV, second growth phase. The cultivation process in the presence of cinnamic acid only showed phase II and phase III. As for the reference, only phase II and phase IV were observed. The results were calculated from biological replicates ($n = 3$) and are given as the mean value ± standard deviation.

amount of acetic acid (220 mM) than the concentration used in the present study resulted in a 36 h lag phase. Yeast cells exposed to the same concentration of formic and levulinic acid exhibited a 72 h and a 69 h lag phase, respectively (Guo and Olsson 2014).

The observed shorter lag phase when applying high initial cell density may find explanation in a recent publication where 38 *S. cerevisiae* strains were compared for their tolerance to acetic acid (Swinnen *et al.* 2014). In that study, the duration of the lag phase was primarily determined by the fraction of cells within the population that resume growth in a strain-dependent manner. The subpopulation that resumes growth after acetic acid exposure was a result of phenotypic cell-to-cell heterogeneity (Swinnen *et al.* 2014). In our case, the total amount of viable cells in the high initial cell density cultivation was 10-fold that of low initial cell density during the lag phase (Fig. 2). It is likely that a higher number of viable cells in high initial cell density culture could increase the likelihood of heterogeneity, and

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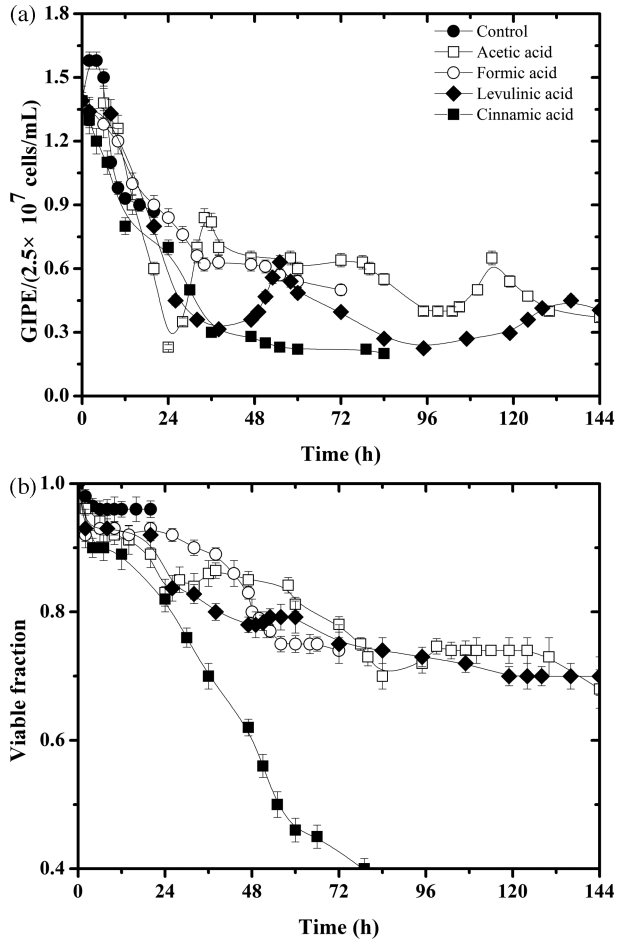

Figure 2. Vitality (**a**) and viable fractions (**b**) of *S. cerevisiae* CEN.PK 113–7D in aerobic cultures without or with addition of 300 mM acetic acid, 220 mM formic acid, 400 mM levulinic acid and 0.8 mM cinnamic acid. The results were calculated from biological replicates ($n = 3$) and are given as the mean value ± standard deviation.

therefore, increase the likelihood that the cells resume growth asynchronously during the acid-adaptation phase, which may have contributed to a shorter lag phase than that of cells at low initial density. In support of this, high initial cell density yielded great variation in the length of the lag phase of yeast cells exposed to different amounts of acetic acid, which were all shorter than that of low initial cell density cultivation (Supplementary Fig. S1).

Effect of increasing initial cell density on the maximum specific growth rate of *S. cerevisiae* CEN.PK

In spite of the observed shorter lag phase in high initial cell density cultivations, the actual maximum specific growth rate on glucose ($\mu_{max\text{-}glu}$) of yeast cells under acetic, formic, levulinic and cinnamic acid was only 27%, 42%, 36% and 15%, respectively, of that of the reference strain (0.26 h^{-1}) (Table 1, Fig. 1). However, yeast cells at the same concentration of formic and levulinic acid exhibited less decrease in $\mu_{max\text{-}glu}$ (50 and 55%, respectively), compared with the control (0.41 h^{-1}) in low initial cell density cultivations (Guo and Olsson 2014). It should be pointed out that because of the presence of dead cells and/or cells in a viable but non-proliferating state, μ_{max} calculated on the total biomass concentration, referred to as apparent specific growth rate (app. μ_{max}) (Vrede 1998), was lower than that of the actual μ_{max} (Supplementary Table S1).

Similarly, the presence of acids also impaired the growth on ethanol. No growth could be observed during the ethanol consumption phase in the presence of cinnamic acid (Fig. 1). The more severe inhibitory effect of the acids on cells during the ethanol growth phase compared with that of cells during the glucose growth phase may have resulted from the low efficiency in energy generation on ethanol compared with that on glucose ($Y_{ATP\text{-}glucose}/Y_{ATP\text{-}ethanol} \approx 2.1$) (Verduyn *et al.* 1991). Therefore, accumulation of cellular acid and acidification of the cytosol may not be avoided (Stratford and Anslow 1998; Ullah *et al.* 2013). Further analysis of the cellular energy level of the acid stressed cells is needed to confirm this.

Accumulation of cells with low metabolic activity under acid stress

In order to better understand the influence of increase in initial cell density on acid-adaptation, cell viabilities, indicated by the fraction of viable cells in total cell counts, were determined. As a complement, the vitalities of cells, reflected by glucose-induced proton efflux (GIPE), were determined by the APT (see Material and Methods) (Fig. 2). The AP test has been developed by Opekarová and Sigler (1982) and it is based on the findings that the ability of yeast cells to excrete protons through the action of membrane H^+-ATPase, H^+/K^+ exchange, and production of CO_2 and organic acids is a strictly controlled process that reflects the metabolic activity of the cells (Sigler, Knotková and Kotyk 1981a; Sigler *et al* 1981b; Sigler, Pascual and Romay 1983). In this assay, water acidification power (WAP) is believed to correlate with the metabolism of endogenous substrates such as glycogen and trehalose, whereas glucose acidification power (GAP) is related to the ability to utilize intra- and extracellular substrates (Siddique and Smart 2000). The determination of WAP and GAP allows the calculation of net GIPE (calculated by subtracting the value of WAP from GAP), which reflects the net efflux of proton across the cell membrane induced only by glucose metabolism.

In the reference cultivation, more than 95% of the yeast cells remained viable during the 20 h cultivation process, and the GIPE value increased up to 1.6 for yeast cells grown on glucose, after which it slowly declined after glucose was depleted (Fig. 2a). However, for cultures where the acids were added, GIPE values decreased greatly during the lag phase (Fig. 2a). After the yeast cells started to grow, the GIPE values increased up to 0.7 and 0.6 for acetic and levulinic acid-stressed cells, respectively. In contrast to observed low yeast vitalities, cell viability was measured to approximately 85% for the cultures where acetic, formic and levulinic acids were added when glucose was present (Fig. 2b). Even though more than 95% of the yeast cells exposed to the same acids remained viable during acid adaptation in low initial cell density cultivations (Guo and Olsson 2014), they only account for 10% of the viable cell fractions in high initial cell density cultivations during the same growth period. Therefore, increasing the initial cell density greatly increased the viable cell fractions in acid adaptation, which may have contributed to a shorter lag phase than that of low initial cell density. However, the viable fractions dropped to 70% as soon as the glucose was exhausted. This is consistent with the previous observation that yeast cells that do not resume growth die after prolonged exposure to acetic acid (Swinnen *et al.* 2014). Moreover, the inhibitory effects of cinnamic acid on yeast cells were more pronounced,

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Table 2. Specific production/consumption rate of *S. cerevisiae* CEN.PK 113–7D in the presence of weak acids at pH 5 under aerobic conditions. The flux analysis was performed for viable cells during exponential growth on glucose. The results were calculated from at least three biological replicates, and are given as the mean values ± standard deviation. N/A: not available.

Specific production/ consumption rate	Reference (no stress)	Acetic acid (300 mM)	Formic acid (220 mM)	Levulinic acid (400 mM)	Cinnamic acid (0.8 mM)
O_2 (mmol g^{-1} h^{-1})	8.5 ± 0.2	6.1 ± 0.2	6.5 ± 0.1	6.3 ± 0.2	6.6 ± 0.3
Glucose (mmol g^{-1} h^{-1})	21.5 ± 0.1	7.9 ± 0.1	9.5 ± 0.2	8.1 ± 0.2	8.9 ± 0.1
CO_2 (mmol g^{-1} h^{-1})	17.8 ± 0.4	11.4 ± 0.3	13.1 ± 0.2	9.2 ± 0.1	14.6 ± 0.2
Ethanol (mmol g^{-1} h^{-1})	28.0 ± 0.3	13.8 ± 0.2	14.8 ± 0.2	14.0 ± 0.2	15.6 ± 0.2
Glycerol (mmol g^{-1} h^{-1})	0.8 ± 0.1	0.3 ± 0.0	0.6 ± 0.1	1.1 ± 0.1	0.3 ± 0.0
Acetate (mmol g^{-1} h^{-1})	0.8 ± 0.0	N/A	0.3 ± 0.0	0.1 ± 0.0	N/A
Pyruvate (mmol g^{-1} h^{-1})	0.4 ± 0.0	0.3 ± 0.0	0.04 ± 0.00	0.3 ± 0.0	0.07 ± 0.00
Succinate (mmol g^{-1} h^{-1})	0.06	0.01 ± 0.00	0.02 ± 0.00	0.02 ± 0.0	0.03 ± 0.00

as can be seen from the substantial decline in viable fraction (as low as 40%) and vitality (about 0.2).

It should be noticed that the AP test, even though it has been widely employed for evaluating the metabolic activity of yeast under different conditions such as starvation, acid washing, dehydration and hyper-osmolality (Sigler *et al.* 2006), has not been used to estimate the changes in cellular physiology caused by acid stress. Comparing the GIPE data with the recently developed methylene blue dye reduction test (Li *et al.* 2011) has confirmed the validity of the AP test to study the vitality of yeast under weak acid stress (Supplementary Fig. S2).

Low vitality resulted in low specific rates of glucose consumption and ethanol production of high initial cell density cultivation

In our previous study, we observed that adding acetic acid, formic acid and levulinic acid at concentrations leading to a reduction of the biomass yield by 50% resulted in 24%, 15% and 16% increase, respectively, in the specific rate of glucose consumption compared with the reference (16.7 mmol g^{-1} h^{-1}), while higher levels of acid slowed down the glycolytic flux in aerobic cultivation of *S. cerevisiae* when using low cell density (Guo and Olsson 2014). Here, we studied the effects on carbon flux to investigate the influence of acid on glycolysis in high initial cell density cultivations.

In general, yeast cells exposed to different acid stress exhibited significant decrease in the specific rates of glucose uptake and ethanol production, largely dependent on the property of the acid, as compared with the reference (Table 2). The low specific rates of glucose consumption and ethanol production for the acid-stressed cells can be largely ascribed to the loss of vitality compared with the reference (as described above). In addition, yeast cells that exhibited higher vitality under acid stress showed higher flux rates. Acetic acid-stressed cells showed a specific glucose uptake rate of 7.9 mmol g^{-1} h^{-1}, and ethanol production rate of 13.8 mmol g^{-1} h^{-1}, which were similar to that of the levulinic acid-stressed cells (8.2 and 14.0 mmol g^{-1} h^{-1}, respectively), and less than that of the formic acid-stressed cells (9.5 and 14.8 mmol g^{-1} h^{-1}, respectively). Yeast cells exposed to cinnamic acid, exhibited higher vitality than that of the cells under stress of the other acids, and showed the highest specific rates of glucose uptake and ethanol production. Similarly, the specific rates of O_2 consumption and CO_2 production significantly decreased as a response to different acid stress. In addition, a 60% decrease in the specific glycerol production rate was observed for acetic, formic and cinnamic acid-stressed cells. It should be noticed again that the presence of fractions of dead cells and/or cells persisting in a viable but non-proliferating state significantly influenced the calculations of flux rates as the values of flux rates calculated on the total biomass concentration were much lower than the values obtained only on viable cells (Supplementary Table S1).

During the second growth phase, the reference culture showed a specific ethanol consumption rate of 3.9 mmol g^{-1} h^{-1}. The specific ethanol consumption rate for formic acid, levulinic acid and cinnamic acid was 1.5, 1.2 and 2.6 mmol g^{-1} h^{-1}, respectively. In addition, formic acid was co-consumed with ethanol at a specific rate of 1.4 mmol g^{-1} h^{-1}. Acetic acid was consumed at a specific rate of 0.5 mmol g^{-1} h^{-1} after ethanol depletion, while yeast was unable to consume levulinic acid.

Exposure to acids caused ATP depletion during the lag phase and low energy level for the growing cells in high initial cell density cultivation

The inhibitory effects of weak acids have been ascribed to uncoupling and anion accumulations, and the counteracting responses of the yeast involve export of proton and anion at the expense of ATP (Stratford and Anslow 1998; Palmqvist and Hahn-Hägerdal 2000b). As a result, glucose uptake is enhanced to facilitate the energy generation (Abbott *et al.* 2007). As we did not observe that acids stimulate glycolysis in high cell density cultivations, the physiological response of yeast cells under acid stress was investigated at cellular energetic level (Fig. 3). We found that yeast cells grown under reference conditions exhibited increased energy charge during growth on glucose compared with growth on ethanol, while the energy charge of acetic, formic and levulinic acid-stressed cells dropped immediately after inoculation (Fig. 3). During both the glucose and ethanol lag phases, the energy charge of yeast cells exposed to levulinic acid was around 0.4, which was significantly lower than that of acetic acid-stressed cells (>0.6). Yeast cells cultured in the presence of formic acid showed higher energy charge than the acetic acid-stressed cells during the glucose lag phase. However, the energy charge dropped dramatically to as low as 0.1 after the glucose growth phase, which was significantly lower than the charge for the cells exposed to acetic acid and levulinic acid. Therefore, the presence of acid caused significant ATP depletion and this effect is more pronounced when the carbon source was depleted. The energy charge of acetic, formic and levulinic acid-stressed cells increased during the exponential growth phase both on glucose and on ethanol, but these values were still lower than the reference.

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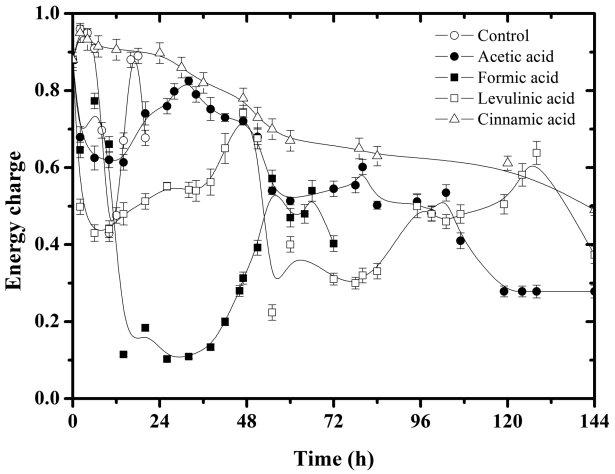

Figure 3. Intracellular energy level of *S. cerevisiae* CEN.PK 113–7D in aerobic cultures without or with addition of 300 mM acetic acid, 220 mM formic acid, 400 mM levulinic acid and 0.8 mM cinnamic acid. The results were calculated from biological replicates ($n = 3$) and are given as the mean value ± standard deviation.

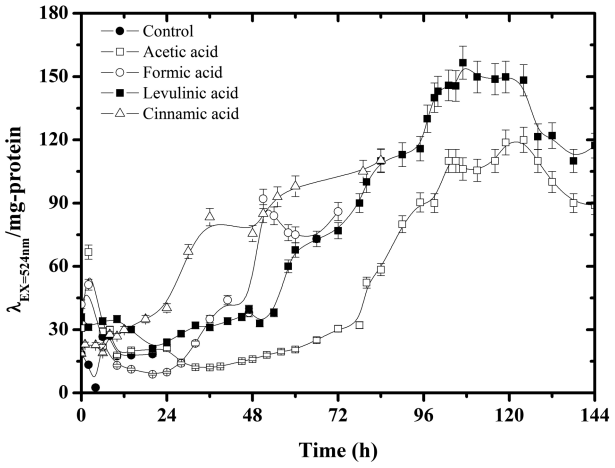

Figure 4. Intracellular oxidation level of *S. cerevisiae* CEN.PK 113–7D in aerobic cultures without or with addition of 300 mM acetic acid, 220 mM formic acid, 400 mM levulinic acid and 0.8 mM cinnamic acid. The results were calculated from biological replicates ($n = 3$) and are given as the mean value ± standard deviation.

Effects of different weak acids on aerobic product formation

In order to investigate acid-specific effects on product formation, the extracellular metabolites were measured during exponential growth phase on glucose. The ethanol yield for yeast cells exposed to formic, levulinic and cinnamic acid increased by 18%, 10% and 25%, respectively, compared with 0.44 C-mol·C-mol^{-1} for the reference cultivation (Table 3). Addition of 300 mM acetic acid, however, led to a 34% decrease in ethanol yield as compared with the reference condition.

For the reference culture, a biomass yield of 0.35 C-mol·C-mol^{-1} was obtained (Table 3). For acetic acid-stressed cells, a final biomass yield of 0.14 C-mol·C-mol^{-1} was calculated by taking into account acetate that had been consumed in addition to glucose, as acetate can be used for biomass synthesis under aerobic condition (van den Berg *et al.* 1996). Slightly lower biomass yield was obtained for levulinic acid-stressed cells compared with the culture supplemented with acetic acid. Addition of 190 mM formic acid produced a biomass yield around 0.17 C-mol·C-mol^{-1}. For cultures to which cinnamic acid was added, the final biomass yield was only 0.05 C-mol·C-mol^{-1}, corresponding to only 14% of the reference. The observed decrease in biomass yield with the addition of weak acids is a physiological response in line with the uncoupling mechanism (Russel 1992). The impact of acid on biomass production was not relieved until the acid was consumed. However, no consumption of formic, acetic and levulinic acids was observed when glucose was present in the cultures, while high initial cell density did enhance the conversion yield of cinnamic acid during the adaption phase (Fig. 1e). Phenylethyl alcohol and dihydrocinnamic acid were detected as the products of cinnamic acid degradation (corresponding to 5% conversion yield of cinnamic acid).

Exposure to acids caused increase in intracellular oxidation level of yeast cells

Weak acids have been shown to trigger endogenous production of superoxide free radicals by affecting the function of the mitochondrial respiratory chain of *S. cerevisiae*, probably by diminishing the proton-motive force of the mitochondria (Kotyk and Georghiou 1991; Piper 1999). In particular, oxidative stress caused by weak acids is enhanced in aerobic yeast cultures due to the activation of the respiratory chain (Piper 1999). The intracellular oxidation level (IOL) of yeast cells grown at reference condition was relatively low (Fig. 4). However, a sharp increase in

Table 3. Comparison of growth and product yields of *S. cerevisiae* CEN.PK 113–7D during aerobic batch growth in the presence of different acids. Biomass yield was calculated as the final biomass produced divided by the substrate consumed. The yields of other metabolites were obtained as the slope of the linear curve when the biomass or metabolite concentration was plotted against the glucose concentration during exponential growth on glucose. The results were calculated from at least three biological replicates, and are given as the mean value ± standard deviation. ND: not detectable.

Yield	Reference (no stress)	Acetic acid (300 mM)	Formic acid (220 mM)	Levulinic acid (400 mM)	Cinnamic acid (0.8 mM)
$Y_{EtOH/S}$ (C-mol·C-mol^{-1})	0.44 ± 0.01	0.29 ± 0.01	0.52 ± 0.00	0.48 ± 0.00	0.55 ± 0.00
$Y_{Gly/S}$ (C-mmol·C-mol^{-1})	18.1 ± 0.1	20.0 ± 0.1	30.3 ± 0.00	52.3 ± 0.0	18.1 ± 0.0
$Y_{Ace/S}$ (C-mmol·C-mol^{-1})	12.3 ± 0.1	ND	11.0 ± 0.0	0.9 ± 0.0	ND
$Y_{Pyr/S}$ (C-mmol·C-mol^{-1})	7.9 ± 0.1	19.3 ± 0.1	2.0 ± 0.0	15.8 ± 0.0	4.4 ± 0.0
$Y_{Suc/S}$ (C-mmol·C-mol^{-1})	2.0 ± 0.1	0.5 ± 0.1	1.1 ± 0.0	1.5 ± 0.0	2.0 ± 0.0
$Y_{X/S}$ (C-mol·C-mol^{-1})	0.35 ± 0.01	0.14 ± 0.01[a]	0.17 ± 0.01	0.09 ± 0.0	0.05 ± 0.00
$Y_{CO2/S}$ (C-mol·C-mol^{-1})	0.18 ± 0.01	0.27 ± 0.02	0.22 ± 0.02	0.18 ± 0.01	0.26 ± 0.02

[a]Based on glucose and acetate consumed.

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IOL was observed after inoculation in cultures containing acetic and formic acid, but not for cinnamic and levulinic acid-stressed cells. After that, a gradual decrease in IOL was observed for yeast cells exposed to acetic acid in lag phase and for the cells grown in the presence of formic acid and levulinic acid, respectively (Fig. 4). Yeast cells exposed to formic acid grown on glucose maintained similar IOL, but yeast cells exposed to cinnamic acid exhibited much higher IOL in comparison with the reference case during the same growth phase. Yeast cells under levulinic acid stress exhibited a higher IOL than acetic acid-stressed cells during the lag phase. Subsequently, the IOL decreased when yeast cells started to grow on glucose (Fig. 4). In general, IOLs dramatically increased after glucose was depleted, and reached the highest level at the end of cultivation.

Exposure to acids caused accumulation of trehalose in yeast cells

The substantial increase in IOL reflected that yeast cells exposed to acids suffered from severe oxidative stress. As trehalose has been shown to protect proteins from oxidative damage and reduce the levels of lipid peroxidation in *S. cerevisiae* during direct oxidative stress (Benaroudj, Lee and Goldberg 2001; Oku *et al.* 2003), trehalose and glycogen content of the yeast cells were monitored to investigate the response of reserve carbohydrate synthesis to acid stress (Fig. 5). Trehalose and glycogen content for the reference cells were less than 2% of cell weight in the whole process of cultivation (Fig. 5a). Exposure of the yeast cells to acids immediately triggered the accumulation of cellular trehalose, especially for acetic acid and levulinic acid-challenged cells, for which the accumulation of trehalose was up to 9% of cell content during the lag phase (Fig. 5a). The cellular trehalose content decreased when yeast cells started to grow on glucose in the presence of acids, and declined to a level similar to the reference when the extracellular carbon source was depleted. Increase in cellular glycogen content was observed only for acetic acid-stressed cells as compared with the reference case. It remains unclear why acetic acid triggers glycogen accumulation, as no evidence has shown a protective role of glycogen under stress conditions (Fig. 5b).

In several biological situations, there are indications that both trehalose and glycogen have an energetic function in yeast cells and their mobilization generally occurs at the onset of glucose depletion (Enjalbert *et al.* 2000). This is different from our observations as degradation of carbohydrate storage happened in the presence of significant amount of glucose. Studies on heat and saline stress on yeast cells have demonstrated mobilization of accumulated trehalose is necessary in growth recovery of the yeast cells (Wera *et al.* 1999; Garre and Matallana 2009). The stored carbohydrates are readily used energy sources (Enjalbert *et al.* 2000), and their mobilization may be necessary to provide energy to support growth resumption after acid adaptation. This assumption may find evidence in a previous report that showed that the ATP surplus produced during the rapid mobilization of reserved carbohydrates supported the budding process (Guillou *et al.* 2004). Interestingly, rapid mobilization of trehalose occurred when the intracellular oxidation levels of the yeast cells increased. Yeast cells with a higher cellular content of trehalose did not show any improvement in the tolerance to acid stress (data not shown). Taken together, these data imply that trehalose may play a role other than protecting yeast cells from acid-induced oxidative stress. Further investigations on the regulatory network of trehalose and glycogen synthesis of the acid-stressed yeast cells are needed to elucidate any significance of accumulation and mobilization of reserve carbon sources for acid tolerance.

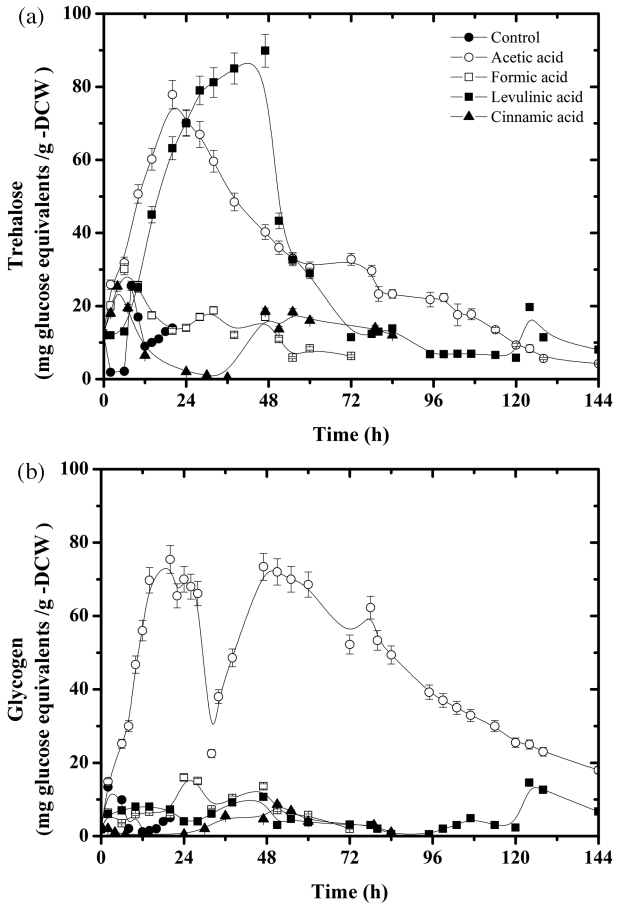

Figure 5. Cellular trehalose (**a**) and glycogen (**b**) content of *S. cerevisiae* CEN.PK 113–7D in aerobic cultures without or with addition of 300 mM acetic acid, 220 mM formic acid, 400 mM levulinic acid and 0.8 mM cinnamic acid. The results were calculated from biological replicates ($n = 3$) and are given as the mean value ± standard deviation.

CONCLUSIONS

Fermentation at high initial cell density led to a reduced lag phase and an increased level of acid that the yeast cells could tolerate under acid stress compared with low initial cell density cultivation. Comparison of physiological parameters (yields, fluxes) demonstrated that *S. cerevisiae* responds differently to acetic, formic, levulinic and cinnamic acids, largely determined by the properties of the acid. All the acids included in our study impaired growth, inhibited glycolytic flux, caused accumulation of trehalose and caused oxidative stress. Trehalose may play a role other than protecting yeast cells from acid-induced oxidative stress. Unlike the other acids, cinnamic acid did not cause depletion of cellular ATP, but exhibited greater inhibitory action on cell function than the other acids even at very low concentration (0.8 mM). Accumulation of glycogen was only observed for acetic acid-stressed cells. Unlike low initial cell density cultivations, exposure to the acids caused significant loss of viability and vitality. The low vitality of yeast cells resulted in low specific rates of glucose consumption and metabolite production.

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SUPPLEMENTARY DATA

Supplementary data are available at FEMSYR online.

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Conflict of interest. None declared.

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