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Evidence for chaotropicity/kosmotropicity offset in a yeast growth model

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Abstract Chaotropes are compounds which cause the disordering, unfolding and denaturation of biological macromolecules. It is the chaotropicity of fermentation products that often acts as the primary limiting factor in ethanol and butanol fermentations. Since ethanol is mildly chaotropic at low concentrations, it prevents the growth of the producing microbes via its impacts on a variety of macromolecular systems and their functions. Kosmotropes have the opposite effect to chaotropes and we hypothesised that it might be possible to use these to mitigate chaotrope-induced inhibition of *Saccharomyces cerevisiae* growth. We also postulated that kosmotrope-mediated mitigation of chaotropicity is not quantitatively predictable. The chaotropes ethanol and urea, and compatible solutes glycerol and betaine (kosmotrope), and the highly kosmotropic salt ammonium sulphate all inhibited the growth rate of *Saccharomyces cerevisiae* in the concentration range 5–15%. They resulted in increased lag times, decreased maximum specific growth rates, and decreased final optical densities. Surprisingly, neither the stress protectants nor ammonium sulphate reduced the inhibition of growth caused by ethanol. Whereas, in some cases, compatible solutes and kosmotropes mitigated against the inhibitory effects of urea. However, this effect was not mathematically additive from the quantification of chao-/kosmotropicity of each individual compound. The potential effects of glycerol, betaine and/or ammonium sulphate may have been reduced or masked by the metabolic production of compatible solutes. It may nevertheless be that the addition of kosmotropes to fermentations which produce chaotropic products can enhance metabolic activity, growth rate, and/or product formation.

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Introduction

Chaotropes are compounds which cause the disordering of other molecular structures (Cray et al. 2013). Of particular biochemical relevance are those which entropically disorder, and can unfold, biological macromolecules such as proteins and nucleic acids

(Bennion and Daggett 2003; Das and Mukhopadhyay 2009; Salvi et al. 2005). Experimentally, the chaotropic properties of compounds such as urea, guanidium hydrochloride and propidium iodide have been widely exploited to denature proteins and in the purification of nucleic acids (Boom et al. 1990; Pace 1986; Van Ness and Chen 1991). Urea is naturally produced by mammals as the end-product of the deamination of amino acids via the urea cycle (Krebs 1942). This avoids the accumulation of highly toxic ammonia, but the urea itself must also be removed by excretion in the urine to avoid excessive build-up. In contrast, kosmotropes promote the ordering of molecules in solution, often resulting in increased rigidity and stability (Kella and Kinsella 1988). Glycerol is often used to protect proteins in solution (e.g. in commercially supplied restriction endonucleases) (Vagenende et al. 2009). This compound, along with proline, betaine and trehalose, is also produced naturally by many micro-organisms as a compatible solute as a response to osmotic stress (Brown 1978, 1990; Brown and Simpson 1972). Although glycerol is not kosmotropic, it has been shown to reduce the adverse effects of ethanol on fungal systems (Bhaganna et al. 2010; de Lima Alves et al. 2015; Hallsworth 1998; Hallsworth et al. 2003). Ammonium sulphate is routinely used for the precipitation and preservation of proteins in the laboratory, an application which relies partly on its kosmotropic properties (Wingfield 1998).

Chaotropicity also has significant implications for industrial fermentations. By far the most common fermentation is the production of ethanol by yeasts or other micro-organisms. This process is required in the production of alcoholic drinks and ethanol-based biofuels. Ethanol is produced naturally by some micro-organisms to inhibit or kill competing species, primarily through its chaotropic effects. However, in fermentations, as the level of ethanol rises it also becomes increasingly inhibitory to the yeasts which produce it. Most industrial strains of *Saccharomyces cerevisiae* can tolerate up to approximately 15%(v/v) ethanol before growth largely ceases and the cells begin to die. Thus, the chaotropicity of ethanol sets upper limits on both the rate of ethanol production and the final ethanol yield which can be produced through batch fermentation (Cray et al. 2015).

The molecular mechanisms of chaotropic activity have not been well-studied for many of the chemically diverse chaotropic stressors, but it is well-known that mechanisms may differ (Ball and Hallsworth 2015; Cray et al. 2013, 2015). It has been suggested that chaotropes such as urea compete effectively for hydrogen bond donors in proteins, thus destabilising secondary structural elements such as α-helices (Bennion and Daggett 2003; Salvi et al. 2005). It has also been assumed that chaotropes act as water-structure breakers for pure solutions of the chaotrope. This assumption is not consistent with the original usage of 'chaotrope' (a substance that disorders biomacromolecules), the experience of the microbial cell, or recent data on the physics of water in the presence of chaotropes (Ball and Hallsworth 2015). Some studies, for example, have suggested that chaotropes increase the overall entropy of the solution, reducing the thermodynamic penalty for the unfolding of proteins (Hatefi and Hanstein 1969; Kresheck and Benjamin 1964; Moelbert et al. 2004; Rupley 1964). Some authors have used molar solution entropies as a proxy measure, but this would only be valid if increased entropy of the system is the principal cause of chaotropicity (Aviram 1973; Miyawaki and Tatsuno 2011). Empirical measures have also been proposed which measure the effects of dissolved compounds on macromolecules. The most-extensive scale utilises the gelation point of agar and spans highly chaotropic compounds such as guanidine hydrochloride to highly kosmotropic compounds such as ammonium sulphate. The method also permits the ranking of different types of compounds including non-polar, barely water-soluble compounds such as benzene, polar organic molecules such as alcohols and ionic compounds such as magnesium sulphate. This scale broadly correlates with other measures, including solution entropies, suggesting common, underlying mechanisms for the various empirical effects observed (Cray et al. 2013). Interestingly, this scale suggests that glycerol behaves unusually. At lower concentration (< 5 M), it is relatively "neutral" on the scale with a molar chaotropicity close to zero. However, at higher concentrations, its molar chaotropicity is comparable to ethanol (Cray et al. 2013).

That it is possible to quantify chaotropicity leads to some intriguing questions. Many other scales are additive, for example, thermodynamic quantities such as free energy, enthalpy and entropy. Even in cases where quantities cannot be added, there are generally ways of calculating the overall value for a mixture

(e.g. the pH scale of acidity and alkalinity). However, the interactions between and within biomacromolecules, water and solutes are complex and dynamic and—for mixtures of solutes—not readily predictable. Furthermore, living cells produce compatible solutes, many of which are kosmotropic (e.g. trehalose), and two of which are chaotropic at sufficient concentration (glycerol and fructose). Therefore, we hypothesized that chaotropicity and kosmotropicity values for individual substances could not be added or subtracted to predict the impacts of solute mixtures on the cellular system. We also postulated that kosmotrope-mediated mitigation of chaotropicity is not quantitatively predictable. Here, we describe experiments to test these postulates by measuring the effects of chaotropes, kosmotropes and mixtures thereof on the growth of yeast in liquid culture.

Materials and methods

Yeast strain, growth media and compounds

S*accharomyces cerevisiae* NCYC 1088 is a non-flocculating ale strain, deposited in the UK National Collection of Yeast Cultures (NCYC) in 1958 by a British Brewery (NCYC 2019). It was stored at 4 °C on Sabouraud Dextrose Agar (SDA) plates (Oxoid) and grown in Yeast Peptone Dextrose broth (YPD broth; yeast extract 10 gl $^{-1}$, bacteriological Peptone from meat 20 gl^{-1}, glucose 20 gl^{-1}; Sigma Aldrich Chemical Company). Ethanol, urea, betaine, ammonium sulphate and glycerol (87%) were all obtained from Sigma Aldrich Chemical Company.

Yeast growth measurements

A single colony of *S. cerevisiae* NCYC 1088 was used to inoculate 10 ml of YPD broth- and incubated overnight at 30 °C with shaking (125 rpm). When an OD_{620nm} of 1 was reached the culture was diluted 1 in 10 with YPD broth and added to a well in a microplate (Thermo Scientific) containing no added compound (control), or glycerol betaine or ammonium sulphate so that the final dilution of yeast was 1 in 100.

The compounds were dissolved in YPD broth-then filter sterilised. Further dilutions were prepared to give a final concentration range of added solutes in the wells of between 2.5 and 15% (v/v for liquids and w/v for solids). Each dilution was tested in triplicate on two to four separate occasions. The microplate was covered in a non-gas permeable film (Thermo Scientific) to prevent evaporation. Growth of the yeast was measured using an Ascent iEMS Multiskan microplate reader (Thermo Lab Systems) at 30 °C with shaking. Optical density measurements at a wavelength of 620 nm were taken every 15 min, for a period of 72 h.

Data analysis

To aid visual analysis, the Weibull growth model (Eq. 1) was applied in GraphPad Prism 6 (GraphPad Software, CA, USA) to the growth curve data.

$$OD = OD_{max} - (OD_{max} - OD_{min}) \exp(-kt)^g \qquad (1)$$

The Weibull distribution is an empirical mathematical model that takes into account the lag, exponential and stationary phases of growth. It is however not able to capture the decline in OD_{600nm} observed at the end of the cultivation; the equation requires adjustment to include a term for cell death (Bevilacqua et al. 2015; Coroller et al. 2006). Growth parameters (lag time, t_{lag}; maximum specific growth rate, μ_{max} and final optical density, OD_{final}) were obtained by fitting the data to the Gompertz Eq. (2) using the Microsoft Excel Add-in DMFit, running under Windows (Gompertz 1825).

$$OD = OD_{min} + (OD_{max} - OD_{min})$$
$$\exp\left(- \exp\left((2.718\mu_{max}/(OD_{max} - OD_{min}))(t_{lag} - t) + 1\right)\right) \qquad (2)$$

For the comparison of growth parameters between controls and experiments, a one-way ANOVA was performed with Dunnet's multiple comparison post hoc test in GraphPad Prism 6.

Results and discussion

In interpreting the growth curves, a number of assumptions were made. Any increase in t_{lag} means that the initial environment is less favourable for growth to begin and the yeast cells had adapt to the conditions of the experiment. Any decrease in μ_{max} means that the environment is less favourable for cell division. This could be because biomacromolecules

are less functional due to unfolding or excessive rigidification. Alternatively, the cells may be forced to divert energy away from cell division and towards cellular homeostasis, e.g. synthesis of heat shock proteins to address protein unfolding. Any decrease in OD_{final} means that the yeast was less able to convert growth media into biomass.

Chaotropes, compatible solutes, and ammonium sulphate inhibit growth at high concentrations

At lower concentrations (up to 2.5%, v/v), ethanol had little effect on the three growth parameters (Fig. 1;

Supplementary Fig. S1). However, above 5% (v/v), t_{lag} was increased and μ_{max} was decreased. At 12.5% (v/v) and 15% (v/v), little or no growth was observed over the course of the experiment (Supplementary Fig. S1). These results were expected and consistent with the well-established inhibitory effects of ethanol on yeast growth. Urea had no significant effect on the growth parameters up to 7.5% (w/v). Above 10% (w/v), it almost completely inhibits the growth such that all three parameters could not be measured (Fig. 1; Supplementary Fig. S1). Glycerol has an inhibitory effect on growth at 10% (v/v), increasing t_{lag} and decreasing μ_{max} (Fig. 2). Of all the solutes tested,

Fig. 1 Summary of growth data in the presence of chaotropes. Each graph summarises the effects of ethanol or urea on the three key growth parameters— t_{lag}, μ_{max} and OD_{max}. For each experimental run, the measurements were obtained in triplicate. Each point represents the mean value resulting from a single experiment. The horizontal line represents the mean of these experimental values and the error bars the standard deviations of these means. Where values were statistically significantly different from the control (no added ethanol or urea), this is shown as: *p < 0.05; **p < 0.01; ***p < 0.001. *nd* not determinable (due to lack of growth under the conditions of the experiment)
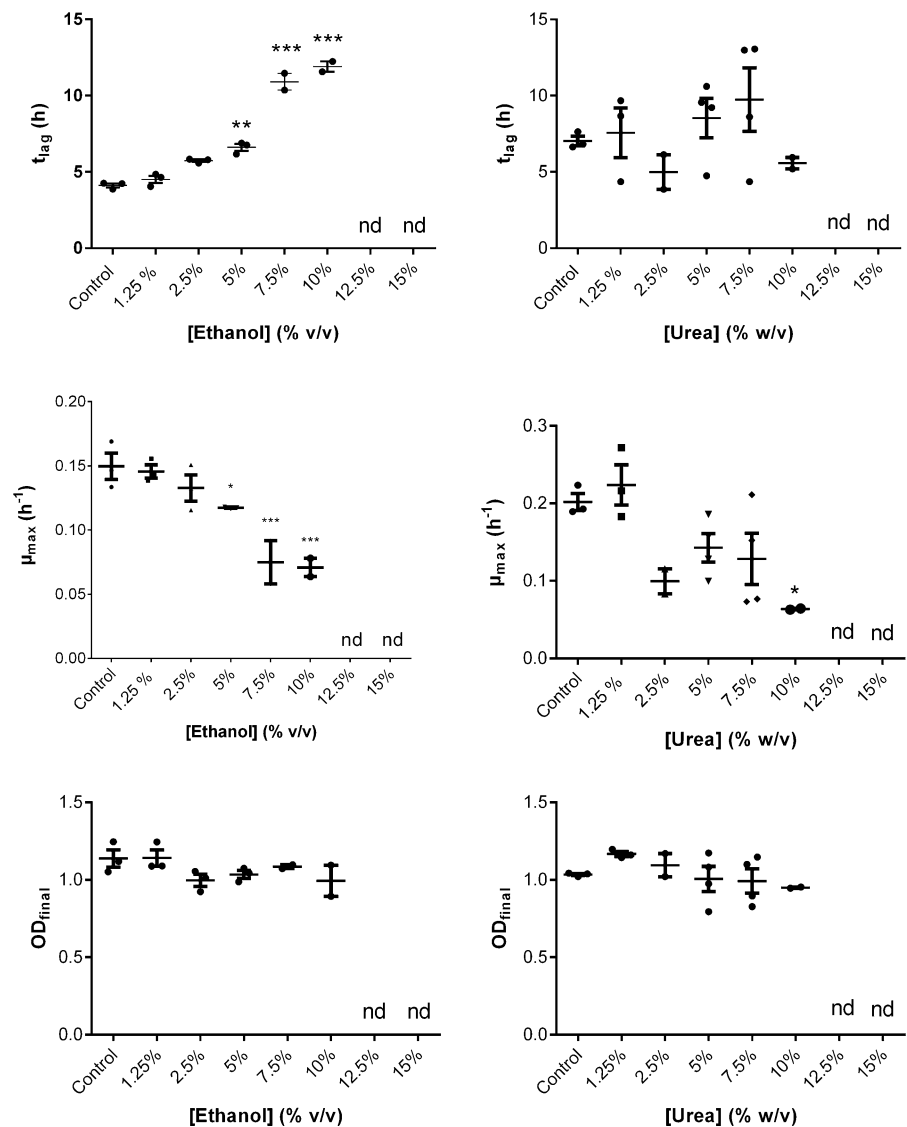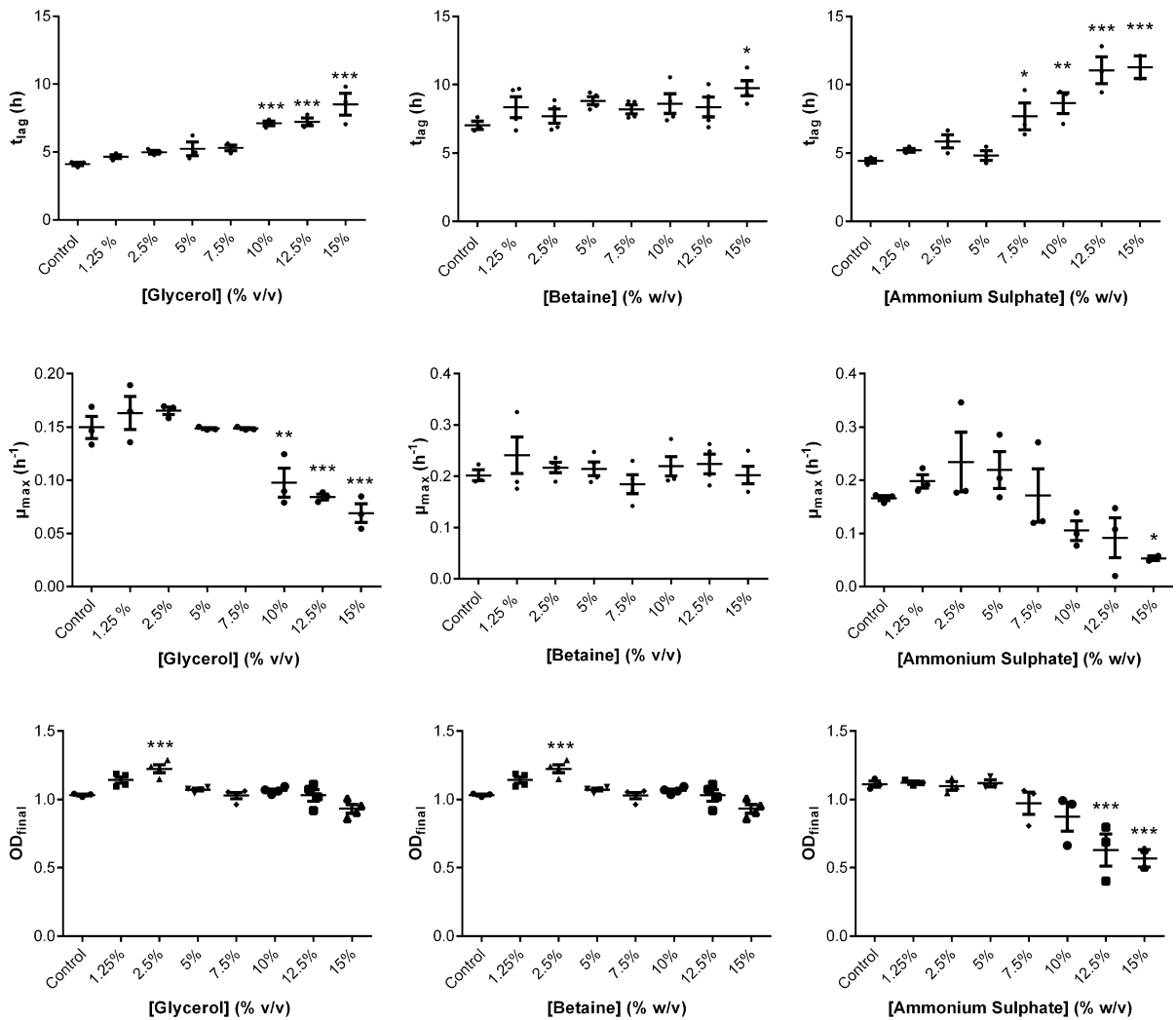
Fig. 2 Summary of growth data in the presence of compatible solutes and kosmotropes. Each graph summarises the effects of glycerol, ammonium sulphate or betaine on the three key growth parameters—t_{lag}, μ_{max} and OD_{max}. For each experimental run, the measurements were obtained in triplicate. Each point represents the mean value resulting from a single experiment. The horizontal line represents the mean of these experimental values and the error bars the standard deviations of these means. Where values were statistically significantly different from the control (no added glycerol, ammonium sulphate or betaine), this is shown as: *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$

betaine had the least effect at the concentrations tested, slightly increasing t_{lag} at 15% (w/v) (Fig. 2; Supplementary Fig. S1). Interestingly, both glycerol and betaine increase the OD_{final} at 2.5% (v/v) and 2.5% (w/v) respectively (Fig. 2; Supplementary Fig. S1). This may result from the utilisation of these compounds as a carbon source by the yeast, or from their roles within the cell as protectants of macromolecular structures against chaotropicity and/or other stresses. At concentrations above 7.5% (w/v), ammonium sulphate increases t_{lag} and above 10% (v/v) decreases OD_{final}

(Fig. 2). This kosmotropic compound has previously been shown to inhibit the growth of the bacterium *Pseudomonas putida* and the filamentous fungus *Fusarium coeruleum* (Bhaganna et al. 2010; Cray et al. 2016). That chao- and kosmotropes can both inhibit growth suggests that cells require optimal flexibility and mobility in their biomolecules: too great an increase in molecular flexibility (which may result in unfolding and the dissociation of supramolecular complexes) or too great in increase in molecular rigidity are both deleterious to the cell. In addition, all
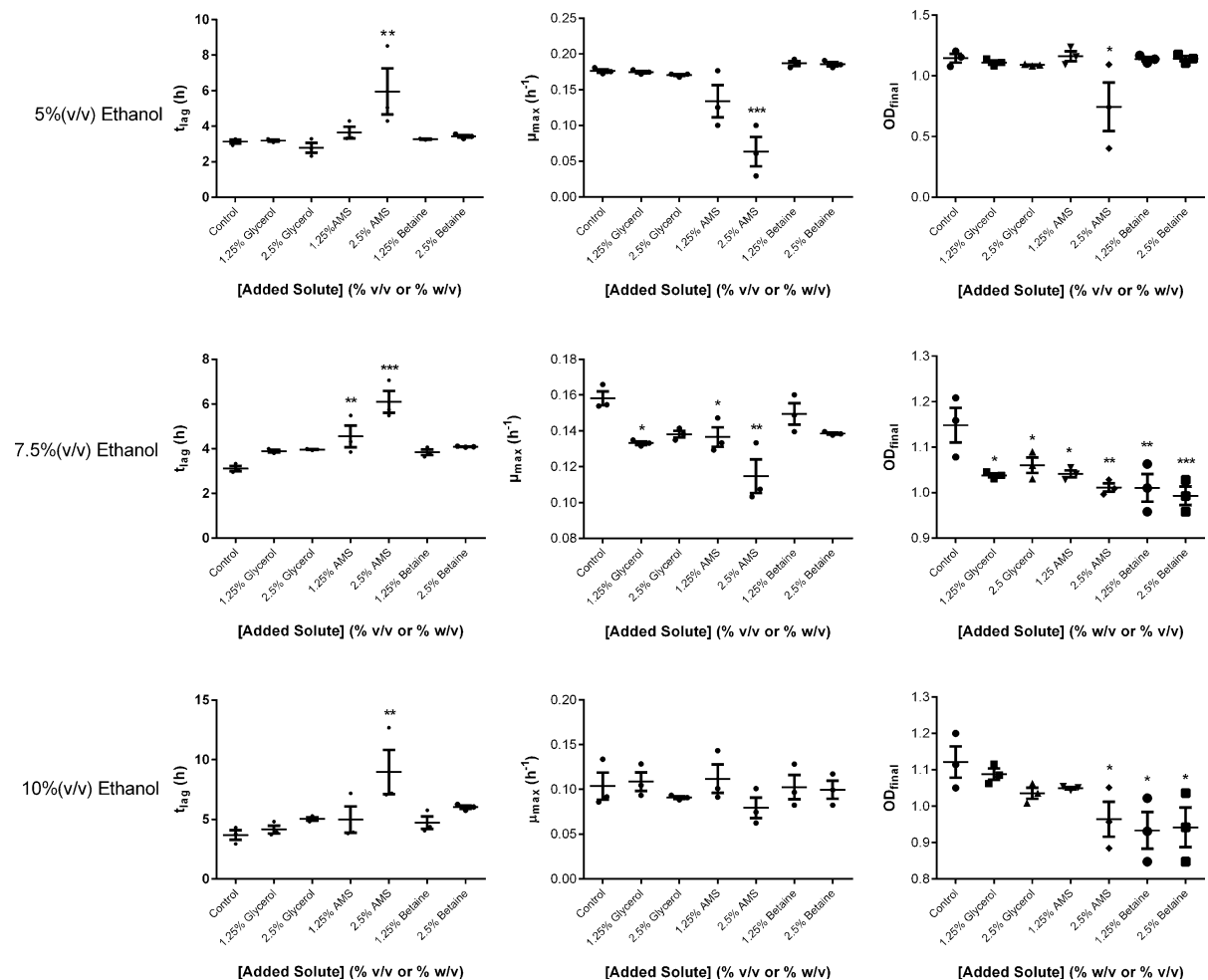
Fig. 3 Summary of yeast growth data in the presence of ethanol and compatible solutes or kosmotropes. Each graph summarises the effects of adding glycerol, ammonium sulphate (AMS) or betaine to yeast cultures growing in the presence of increasing concentrations of ethanol. For each experimental run, the measurements were obtained in triplicate. Each point represents the mean value resulting from a single experiment. The horizontal line represents the mean of these experimental values and the error bars the standard deviations of these means. Where values were statistically significantly different from the control (no added glycerol, ammonium sulphate or betaine), this is shown as: $*p < 0.05$; $**p < 0.01$; $***p < 0.001$

of the compounds tested also reduce water activity, and some of them (betaine and ammonium sulphate) cause osmotic stress, so chao-/kosmotropic-effects do not operate in isolation. It may also be that some of the added compounds are assimilated as a nutrient source. Ethanol, glycerol and betaine can all act as carbon sources in some *S. cerevisiae* strains. However, the data deposited at NCYC on this strain suggests that, while ethanol can be utilised, glycerol cannot; there is no data on betaine (NCYC 2019). Ammonium sulphate can act as a nitrogen source. Thus, any inhibitory effects of these compounds might be partly offset by their nutritional benefits. Nevertheless, these results suggest that increasing the chaotropicity or the kosmotropicity of the media tends to inhibit yeast growth.

Compatible solutes and ammonium sulphate did not mitigate against inhibition of growth under the conditions tested

The effects of glycerol, betaine and ammonium sulphate were assessed at three ethanol concentrations—5, 7.5 and 10% (v/v). These values were chosen
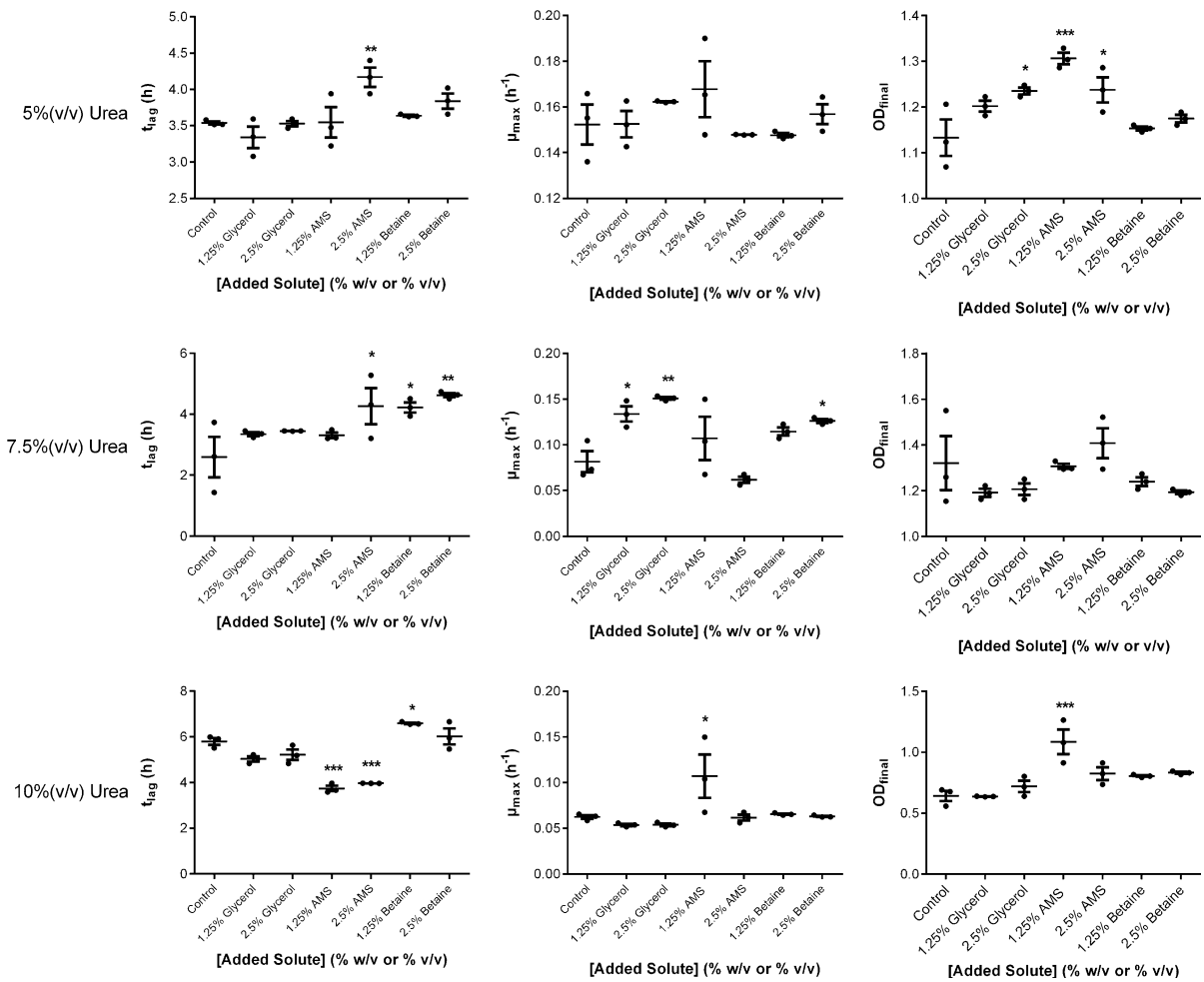
Fig. 4 Summary of yeast growth data in the presence of ethanol and compatible solutes or kosmotropes. Each graph summarises the effects of adding glycerol, ammonium sulphate (AMS) or betaine to yeast cultures growing in the presence of increasing concentrations of urea. For each experimental run, the measurements were obtained in triplicate. Each point represents the mean value resulting from a single experiment. The horizontal line represents the mean of these experimental values and the error bars the standard deviations of these means. Where values were statistically significantly different from the control (no added glycerol, ammonium sulphate or betaine), this is shown as: *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$

since they have a clear effect on the growth parameters, but are sub-lethal and did not completely inhibit growth (Fig. 1; Supplementary Fig. S1). At all three ethanol concentrations, glycerol (1.25%, v/v and 2.5%, v/v), betaine (1.25%, w/v and 2.5%, w/v), and ammonium sulphate (1.25%, w/v and 2.5%, w/v) did not improve the growth parameters (Fig. 3; Supplementary Fig. S2). In some cases, ammonium sulphate caused a further deterioration in these parameters, increasing t_{lag}, decreasing μ_{max} and decreasing OD_{final} when compared to ethanol only controls (Fig. 3). At high concentrations, the chaotropicity of glycerol may act synergistically with the chaotropicity of ethanol;

the osmotic stress induced by betaine may be inhibitory, and the ionic nature and/or kosmotropic activities of ammonium sulphate may act to impair yeast growth.

Compatible solutes and ammonium sulphate can mitigate against yeast growth inhibition by urea

Cultures grown in the presence of urea, typically had improved growth profiles in the presence of ammonium sulphate, glycerol or betaine. Growth curves were shifted leftwards and upwards compared to controls in urea only (Supplementary Fig. S2). There

were varying effects on the growth parameters (Fig. 4). These depended on the added compound and the concentration of urea which varied from 5, 7.5 and 10%, w/v corresponding to molar concentrations of 0.83, 1.25 and 1.66 M, and to chaotropicities of 13.8, 20.8 and 27.6 kJ kg^{-1} (Cray et al. 2013), respectively. In some cases, but not all, they partially alleviated the effects of urea on the growth parameters. Ammonium sulphate reduced t_{lag} at 10% (w/v) urea, but not at lower concentrations of the chaotrope (Fig. 4). At 7.5% (w/v) urea, glycerol (at 1.25%, v/v and 2.5%, v/v) and betaine (2.5%, v/v) both partially offset the effects on μ_{max} when compared to urea only controls (Fig. 4). At 5% (w/v) urea, glycerol (2.5%, v/v); net chaotropicity 14.1 kJ kg^{-1}) and ammonium sulphate (1.25%, w/v) and 2.5% (v/v); offset the reduction of OD$_{final}$ (Fig. 4). While no added compound was able to completely offset the effects of urea at any of the concentrations tested, these results nevertheless demonstrate mitigation against chaotropicity. As predicted, the effects of chao- and kosmotropicity are not additive for this yeast model.

Conclusions

While the addition of kosmotropes can mitigate chaotropicity in some cases, the effect is not quantitatively predicted in this yeast model. This is broadly similar to the results observed in an isolated enzyme model. Here, both chaotropes and kosmotropes depressed the activity of the enzyme and combinations only partially restored activity in a minority of cases (Bell et al. 2013). However, relatively low concentrations of compatible solutes were used in this study, relative to those which can be found in microbial cells under stress. If we had made an assumption that there is a linear relationship between these parameters and that chaotropicity (or kosmotropicity) in a mixture of compounds is additive, the current study would have disproved this. Some work in which chaotropicities have been determined empirically suggests that the relationships may be more complex (de Lima Alves et al. 2015; Fox-Powell et al. 2016; Yakimov et al. 2015). Further work to understand these relationships is necessary to inform quantitative studies on the mitigation of chaotropicity in biofuel production and other fermentations.

Furthermore, in the yeast growth model, the effects which were observed only applied in the case of urea: we observed no mitigation in the case of ethanol. The reasons for this will require further investigation. For example, in addition to being chaotropic, ethanol reduces water activity, although previous work has demonstrated that the mode of action of both compounds at low-to-moderate concentrations is chaotropic (de Lima Alves et al. 2015).

Mitigation by compatible solutes and kosmotropes against chaotrope-induced stresses have been reported in numerous studies of enzyme- and cellular systems [e.g. (Bhaganna et al. 2016; Bhaganna et al. 2010; Chin et al. 2010; Cray et al. 2016; Cray et al. 2015; de Lima Alves et al. 2015; Hallsworth 1998; Hallsworth et al. 2003; Hallsworth et al. 2007; La Cono et al. 2019; Stevenson et al. 2015; Stevenson et al. 2017; Williams and Hallsworth 2009; Yakimov et al. 2015)]. Many of these studies carried out "testing-to-destruction" where enzyme or cellular systems were exposed to chaotropicity at the edge of their window for tolerance. Under these extreme circumstances, kosmotropic compatible solutes and other kosmotropic substances, as well as glycerol, mitigated against chaotropicity. It may be that the *S. cerevisiae* cell produces sufficient compatible solutes under moderate stresses that exogenous compounds are not required or effective. Furthermore, it may be that some of the compounds added in the current study were utilised as nutrients (see above). Nevertheless, the data presented here do suggest that mitigation of chaotropicity during fermentations may be worth considering where this is a limiting factor. Future studies should concentrate on high ethanol concentrations where chaotropicity can induce cell-system failure.

Supporting information Supplementary Figure S1—Effects of chaotropes, kosmotropes and compatible solutes on the growth of *S. cerevisiae* NCYC 1088. These growth curves were used to derive the growth parameters shown in Figures 1 and 2.

Supplementary Figure S2—Effects of kosmotropes and compatible solutes on the growth of *S. cerevisiae* NCYC 1088 when challenged with ethanol or urea. These data were used to obtain the growth parameters shown in Figures 3 and 4.

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