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Destressing yeast for higher biofuel yields: Can excess chaotropicity be mitigated?

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Please select a section/category for your manuscript.	Biofuels and Biochemicals from Renewable Bioresources - Renewable feedstocks, especially the non-food biomass resources. The section includes novel microbes, enzymes, processes involved in the production of biofuels and biochemicals from renewable bioresources with the focus on non-food biomass resources.
Abstract:	Biofuels have the capacity to contribute to carbon dioxide emission reduction and to energy security as oil reserves diminish and/or become concentrated in politically unstable regions. However, challenges exist in obtaining the maximum yield from industrial fermentations. One challenge arises from the nature of alcohols. These compounds are chaotropic (i.e. causes disorder in the system) which causes stress in the microbes producing the biofuel. Brewer's yeast (Saccharomyces cerevisiae) typically cannot grow at ethanol concentration much above 17%(v/v). Mitigation of these properties has the potential to increase yield. Previously, we have explored the effects of chaotropes on model enzyme systems and attempted (largely unsuccessfully) to offset these effects by kosmotropes (compounds which increase the order of the system, i.e. the "opposite" of chaotropes). Here we present some theoretical results which suggest that high molecular mass polyethylene glycols may be the most effective kosmotropic additives in terms of both efficacy and cost. The assumptions and limitations of these calculations are also presented. A deeper understanding of the effects of chaotropes on biofuel-producing microbes is likely to inform improvements in bioethanol yields and enable more rational approaches to the "neutralisation" of chaotropicity.

Reviewer #1: 1、The references were not numbered in the order they appear. For example, references 1 and 2 first appeared in the fourth paragraph of the text. Reference (15, 24, 25) appears in the 'Introduction' section.

We apologise for this error and have now used the correct endnote template.

2. The language in the text needs to be carefully polished.

We have read the paper carefully and made a number of corrections.

3. The title of the article should be further specified.

We agree. The title has been changed to "Destressing yeast for higher biofuel yields: Can excess chaotropicity be mitigated?"

4. The paragraph structure of the article is suggested to be adjusted. For example, the author mainly talked about 'chaotropes' and 'kosmotropes', and glycerol does not belong to the typical 'chaotropes' or 'kosmotropes'. It seems to be incompatible as a paragraph 'The problem with glycerol 'alone.

We agree. We have amalgamated this short section into the preceding one.

5. The authors mentions that'Bioethanol fermentations have a theoretical maximum yield of around 17% (v/v) ethanol (17, 30)'. It seems that 20% (v/v) has been reached, please refer to the article [30] in your reference list.

We thank the reviewer for this valid point. We have modified the text accordingly.

"Bioethanol fermentations have a theoretical maximum yield of around 17%(v/v) ethanol, under conditions similar to those used industrially (i.e. around 30°C). Yields of up to 20% (v/v) in sake fermentations which are carried out at low temperatures, over extended periods of time and with specially selected yeast strains."

6. The author present some theoretical results which suggest that high molecular mass polyethylene glycols may be the most effective kosmotropic additives in terms of both efficacy and cost. It is recommended to state the effect of adding PEG (such as 0.023 M PEG6000) on the growth of Saccharomyces cerevisiae itself and ethanol fermentation.

The referee raises a valid point. High molecular mass PEGs may exert osmotic effects on cells. We have added a sentence to note this.

"However, high molecular mass PEGs may exert osmotic effects on the yeast cells which may reduce growth rates and ethanol yields. Experimental testing of these additives is recommended."

7. The author states that it may be possible to regulate the chaotropicity by addition of kosmotropic solutes. It is recommended that the author list a table to clearly show that the mitigation of product chaotropicity by provision of exogenous kosmotropic substances in others' research works.

We think this is an excellent idea and have incorporated this as new table 1.

8. Due to the presence of various inhibitors, the concentration of ethanol is often not high in the production of cellulosic ethanol. Do these inhibitors also have chaotropicity? Will they interfere with the use of kosmotropic additives?

The reviewer raises an important point. While sugars such as glucose are relatively "neutral" on the chaotropicity scale, some other compounds which may be present (e.g. phenols and vanillin) are chaotropic. We have noted this in the paper.

"Although fermentation substrates, typically sugars such as glucose and sucrose, are relatively "neutral" on the chaotropicity scale, other compounds which may be present in feedstocks (e.g. phenols and vanillin) are chaotropic. The presence of these additional chaotropes may need to be considered in any mitigation strategy."

Commentary:

Destressing yeast for higher biofuel yields: Can excess chaotropicity be mitigated?

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Footnote: This commentary is based in part on a presentation given at The International Conference on Energy and Sustainable Futures (ICESF), Nottingham, UK, in September 2019.

Abstract

Biofuels have the capacity to contribute to carbon dioxide emission reduction and to energy security as oil reserves diminish and/or become concentrated in politically unstable regions. However, challenges exist in obtaining the maximum yield from industrial fermentations. One challenge arises from the nature of alcohols. These compounds are chaotropic (i.e. causes disorder in the system) which causes stress in the microbes producing the biofuel. Brewer's yeast (*Saccharomyces cerevisiae*) typically cannot grow at ethanol concentration much above 17%(v/v). Mitigation of these properties has the potential to increase yield. Previously, we have explored the effects of chaotropes on model enzyme systems and attempted (largely unsuccessfully) to offset these effects by kosmotropes (compounds which increase the order of the system, i.e. the "opposite" of chaotropes). Here we present some theoretical results which suggest that high molecular mass polyethylene glycols may be the most effective kosmotropic additives in terms of both efficacy and cost. The assumptions and limitations of these calculations are also presented. A deeper understanding of the effects of chaotropes on biofuel-producing microbes is likely to inform improvements in bioethanol yields and enable more rational approaches to the "neutralisation" of chaotropicity.

Keywords: Bioethanol; kosmotropicity; chaotropicity; fermentation; biofuel yield; bioenergy

1. Introduction

Biofuels have the potential to replace fossil fuels in many applications. They offer considerable environmental advantages over fossil fuels since they are truly renewable and have lower overall net carbon dioxide emissions. Since the normal precursors are either crop plants or organic waste, these can be produced locally reducing the need to transport fuels over long distances. This has consequent benefits for the environment, the cost of the fuel and for energy security [1-3]. However, there are several problems with biofuels which may prevent their more widespread adoption. The reliance on crop plants means that there is the potential for competition between food and fuel uses of crops and the land used to produce them [1,4]. There are also several challenges in achieving high yields. These relate, in part, to difficulties in digesting some plant matter, notably celluloses and lignins [1,5]. This means that a substantial fraction of the carbon in the plants is not readily converted to fuel. The fuels themselves often inhibit their own biosynthesis, by "poisoning" the microbes which are producing them. This "toxic" effect has a variety of causes; a key issue is the chaotropicity of compounds commonly used as biofuels, e.g. ethanol and butanol[6] . This is recognised as a significant, limiting factor in maximising biofuel yields [7]. However, to improve the environmental and economic attractiveness of biofuels, yields need to rise. This commentary focusses on the mitigation of chaotropicity in the production of ethanol by the baker's or brewer's yeast, *Saccharomyces cerevisiae*. Many of the issues considered will also apply to other biofuel fermentation processes.

2. Chaotropes and kosmotropes of relevance to biofuel fermentations

Chaotropes are compounds which increase the overall entropy of a solution [8]. This has particular relevance in biology since this results in the disordering and unfolding of macromolecules and the disruption of biological membranes [9,10]. Since cells rely on membranes to define their various compartments and biological macromolecules depend on their three-dimensional conformations for their correct activities and functions, chaotropes often cause generalised, non-specific toxicity to living systems. The molecular basis of chaotropicity remains uncertain, somewhat controversial and may vary with the chaotrope and the system being disrupted [11]. Chaotropes cause disruption of the hydrophobic interactions which stabilise proteins, DNA and membranes. This is partly due to the increased system entropy reducing the entropic penalty for exposing hydrophobic residues to the bulk water and may also result from specific interactions between the chaotropic molecule and functional groups within the macromolecule [8,12]. Kosmotropes have the opposite effects. They reduce solution entropy and promote the ordering and rigidification of biological macromolecules.

The quantification of chaotropicity has not been straightforward. Given the links to entropic changes, entropies of solvation often correlate with the effects observed on phenomena such as protein stability [8]. However, the most extensive quantitative scale of chaotropicity available to date is based on an empirical measure, changes to the gelling temperature of agar. This scale can be used with almost any water-soluble compound and has been applied to salts, small organic molecules and macromolecules such as (poly)ethylene glycol (PEG). It spans a wide range of values of chaotropicity (positive) and kosmotropicity (negative). Values around zero are considered to be "neutral" [8].

The products of biofuel fermentations are typically chaotropic, for example ethanol (molar chaotropicity 5.93 kJ kg^{-1} mol^{-1}) and butanol (37.4 kJ kg^{-1} mol^{-1}). Although fermentation substrates, typically sugars such as glucose and sucrose, are relatively "neutral" on the chaotropicity scale, other compounds which may be present in feedstocks (e.g. phenols and vanillin) are chaotropic [8]. The presence of these additional chaotropes may need to be considered in any mitigation strategy [7]. Microbial cells naturally mitigate the effects of chaotropicity by producing compatible solutes, many of which are kosmotropes. These include trehalose (molar chaotropicity, -10.6 kJ kg^{-1} mol^{-1}), betaine (-25.5 kJ kg^{-1} mol^{-1}), proline (-5.8 kJ kg^{-1} mol^{-1}) and glycerol (1.1 kJ kg^{-1} mol^{-1}) [8]. This raises the interesting hypothesis that it may be possible to regulate the chaotropicity of biofuel fermentations by the addition of kosmotropic solutes. This would be analogous to the regulation of the pH in fermentations by the addition of acids and bases. Ideally it would be possible to predict the effects of kosmotrope addition (just as it is with acid/base addition). To do this, it is necessary to understand how to calculate the net chaotropicity of a mixture of chaotropic and kosmotropic compounds.

Although glycerol is commonly produced by microbes as a compatible solute, it is not a kosmotrope. On the agar gel point scale, it is close to "neutral" at moderate concentrations (<5 M) and more chaotropic at higher concentrations [8]. This suggests that its mode of action is not through the direct "neutralisation" of chaotropicity, but perhaps through more direct interactions which stabilise biological macromolecules. It also suggests that its chaotropicity is not a linear function of its concentration. While this effect has not been observed with other compounds, the limited state of our knowledge means that this possibility cannot be ruled out. Non-linear relationships between concentration and chaotropicity would considerably complicate any calculations of net chaotropicity and thus the practicalities and economics of applying this in commercial biofuel fermentations.

4. Quantification of chaotropicity – some assumptions and conclusions of relevance to biofuels

When calculating net chaotropicities, we made two initial assumptions. First, we assumed that there is a linear relationship between chaotropicity and concentration. This means that the chaotropicity of any concentration of a compound can be readily calculated from the molar chaotropicity. We also assumed that chaotropicities (and kosmotropicities) are additive. In other words, if we have two compounds in solution, with one compound contributing a chaotropicity of X kJ kg^{-1} and the other Y kJ kg^{-1}, the net solution chaotropicity should be X+Y kJ kg^{-1}. This follows from the assumption of linearity of the relationship between chaotropicity and concentration. It is based on an underlying assumption that the molecular mechanism of chaotropicity is essentially the same for all compounds. It also assumes no significant interactions between the two types of molecule in solution which might affect their chaotropic effects.

Bioethanol fermentations have a theoretical maximum yield of around 17%(v/v) ethanol, under conditions similar to those used industrially (i.e. around 30°C) [13,14]. Yields of up to 20% (v/v) in sake fermentations which are carried out at low temperatures, over extended periods of time and with specially selected yeast strains [15]. At these concentrations yeast cells cease to function, partly due to the chaotropicity of ethanol. However, actual yields are typically lower, for example [16-22]. Yeast cells are remarkably well adapted to functioning in relatively high ethanol concentrations compared to most microbes [3,23,24]. Thus, *S. cerevisiae* can be classified as a zymogenous species, i.e. one which grows well on substrates which are readily available in the environment and easily metabolised [25-27]. Recent work has suggested that stress should not always be considered harmful for microbes since it drives vitality and genetic diversity [28,29]. Thus, it is possible to select for strains with higher ethanol tolerance [3]. Fermentation processes can be designed to mitigate stress. For example, temperature and pH can be carefully controlled, excess ethanol can be removed, and growth media optimised [30]. We propose that chaotropicity mitigation may also be helpful and we summarise some examples of this in table 1.

Table 1: Examples of the mitigation of chaotropicity by kosmotropes

Chaotrope	**Kosmotrope**	**Comments**	**References**
Urea	Trimethylamine *N*-oxide	Used by elasmobranch fish to mitigate the toxic effects of urea.	[31]
Urea	Betaine or ammonium sulphate	Partially mitigate effects on yeast growth in a laboratory study.	[32]
Ethanol	Trehalose	Produced by many microorganisms, including yeast to mitigate chaotrope stress.	[33,34]

Ethanol	Ectoine	Partially mitigates chaotropicity in fermentations by *Zymomonas mobilis.*	[35]
Ethanol	Proline	Mitigates chaotropic stress in many microorganisms, including yeast.	[36]
tert-Butyl alcohol	Trimethylamine *N*-oxide	Chaotropicity "neutralised" in theoretical and laboratory studies.	[37,38]
Butanol	Proline	Engineering *Bacillus subtilis 168* to increase proline production increased butanol yield.	[39]

A concentration of ethanol of 17%(v/v) is equal to a molar concentration of 2.9 M and, therefore, to a solution chaotropicity of 17.2 kJ kg^{-1}. To return this value to "neutral" would, assuming that chaotropicities are additive, require the addition of a kosmotropic compound at a concentration which has a chaotropicity of -17.2 kJ kg^{-1}. This could be achieved by adding 0.26 M ammonium sulphate, 0.68 M betaine, 2.9 M proline, 1.1 M PEG 200, 0.14 M PEG 1000 or 0.023 M PEG 6000. However, experimental investigations suggest that the situation is more complex. Attempts to offset the chaotropic effects of alcohols on the enzyme β-galactosidase were largely unsuccessful. Indeed, when used on their own, all of the kosmotropes tested also inhibited the enzyme to similar extents to the chaotropic alcohols [40]. Similar results have been obtained in a yeast model in which the effects of chaotropes, kosmotropes and mixtures thereof on growth were tested [32]. Other studies also question the additivity of chaotropicities in real biological situations, for example [41-44]. These all demonstrate complex relationships where the chaotropicites of mixtures were measured directly using the agar gelation method. Nevertheless, these studies also broadly support the hypothesis that kosmotropes can offset the detrimental effects of chaotropes. Unfortunately, in a yeast model, while the effects of urea could be partially offset by ammonium sulphate and betaine, no equivalent effects were observed with ethanol [32]. The reasons for this difference are currently unknown.

5. **The economics of kosmotrope addition**

If kosmotropes are to be added to biofuel fermentations, it will need to be economically as well as scientifically viable. There would be little point in adding expensive, additional reagents for a marginal increase in yield. This means that we need to consider the cost per unit kosmotropicity (Table 2). This calculation suggests that ammonium sulphate or PEG 6000 would be the best additives to consider in commercial fermentations. Given that ammonium sulphate addition would raise the ionic strength of the fermentation mix, the use of electrically neutral PEG might be preferred. However, high molecular mass PEGs may exert osmotic effects on the yeast cells, which may reduce growth rates and ethanol yields. Experimental testing of these additives is recommended. It should be noted that this calculation is based on current prices (with no allowance for commercial pricing or deals for large orders) and further assumes (unrealistically) that prices would be unchanged in the event of considerably increased demand for a compound from the biofuel industry. Nevertheless, the rankings presented here are likely to be broadly correct.

Table 2: Some costs per unit kosmotropicty of compounds of relevance to the biofuel industry

Compound	**Molar chaotropicty**[a] **kJ kg^{-1} mol^{-1}**	**Cost per unit kosmotropicity**[b] **(£ per kJ kg^{-1} mol^{-1})**

Betaine	-25.5	0.58
Proline	-5.8	11.35
Trehalose	-10.6	46.89
Ammonium sulphate	-66.9	0.14
PEG 200	-15.0	0.84
PEG 1000	-126	0.29
PEG 6000	-659	0.16

Notes:

[a] Values from ref [8]; a negative value for chaotropicity represents a kosmotropic compound.

[b] Prices from Sigma-Aldrich price list (www.sigmaaldrich.com/catalog/) as of 28th April 2019.

6. (Currently) unanswered questions

In addition to the problems noted above with the quantification of mixtures of chaotropes and kosmotropes, there are some other areas which require further elucidation. A greater understanding of the molecular basis of chaotropicity and kosmotropicity is required from experimental and *in silico* studies. The mode of action of glycerol also requires greater understanding. How ethanol's chaotropic properties interact with its mildly hydrophobic properties needs to be explained.

Critically a fuller understanding the relationship between chaotropicity and concentration is required along with robust methods to estimate net chaotropicity of mixtures. While thermodynamic properties (e.g. enthalpy and entropy) are additive, some other chemical properties are not. For example, while the pH of mixtures can be predicted using pK_a values and concentrations, pH values are not additive. Alternatively, a method to measure the net solution chaotropicity experimentally would circumvent the need for calculating this value. This would require the invention of a chaotropicity meter, analogous to instruments which measure pH or ionic strength. No such instrument has been designed, but it would need to be reusable in order to be economically attractive to the biofuel industry. The agar gel point method covers a wide range of chao- and kosmotropicity values and is applicable to different types of compounds [8]. However, as currently implemented, it is not reusable because the solution being tested is mixed with the agar. Therefore, a completely new method may be required (e.g. one based on the unfolding/folding of a protein, or a biophysical measurement such as nanorheology [45]).

7. **Conclusion**

There is scope to use kosmotropes as additives to mitigate the chaotropic effects observed in biofuel fermentation. However, much greater understanding of the mechanism of chaotropicity and the quantification of this phenomenon is required before this can be done rationally. Until then, it may be possible to develop empirical, “trial and error” methods which are specific to individual fermentation conditions.

Acknowledgments

JE thanks the University of Brighton and the Universities Alliance Doctoral Training Alliance for a PhD studentship. DJT acknowledges his long-standing collaboration with Dr John E Hallsworth (Queen’s University, Belfast, UK) who first encouraged him to think about problems of chaotropicity in cellular systems. We thank Dr Samantha Banford for her assistance with the revised version of this paper.

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