The Roles and Applications of Chaotropes and Kosmotropes in Industrial Fermentation Processes

David J. Timson[1,*]

[1] School of Pharmacy and Biomolecular Sciences, University of Brighton, Huxley Building, Lewes Road, Brighton, BN2 4GJ. UK

*Corresponding author (email: d.timson@brighton.ac.uk)

Abstract

Chaotropicity has long been recognised as a property of some compounds. Chaotropes tend to disrupt non-covalent interactions in biological macromolecules (e.g. proteins and nucleic acids) and supramolecular assemblies (e.g. phospholipid membranes). This results in the destabilisation and unfolding of these macromolecules and assemblies. Unsurprisingly, these compounds are typically harmful to living cells since they act against multiple targets, comprising cellular integrity and function. Kosmotropes are the opposite of chaotropes and these compounds promote the ordering and rigidification of biological macromolecules and assemblies. Since many biological macromolecules have optimum levels of flexibility, kosmotropes can also inhibit their activity and can be harmful to cells. Some products of industrial fermentations, most notably alcohols, are chaotropic. This property can be a limiting factor on rates of production and yields. It has been hypothesised that the addition of kosmotropes may mitigate the chaotropicity of some fermentation products. Some microbes naturally adapt to chaotropic environments by producing kosmotropic compatible solutes. Exploitation of this in industrial fermentations has been hampered by scientific and economic issues. The cost of the kosmotropes and their removal during purification needs to be considered. We lack a complete understanding of the chemistry of chaotropicity and a robust, quantitative framework for estimating overall chaotropicities of mixtures. This makes it difficult to predict the amount of kosmotrope required to neutralise the chaotropicity. This review considers examples of industrial fermentations where chaotropicity is an issue and suggests possible mitigations.

Keywords: biofuels; ectoine; entropy; ethanol; fermentation; urea; vanillin

Introduction: Chaotropicity and Kosmotropicity

Chaotropes are compounds which induce disorder in biological macromolecules and supramolecular assemblies (Cray et al. 2013). Thus, they tend to unfold the three-dimensional structures of proteins and nucleic acids and disrupt phospholipid membranes (Bennion and Daggett 2003; Das and Mukhopadhyay 2009; Van Ness and Chen 1991). Mole for mole, some chaotropes are more disordering than others and, therefore, we recognise stronger and weaker chaotropes. In the laboratory, urea and guanidinium salts are commonly used to denature proteins and nucleic acids. Kosmotropes are the "opposite" of chaotropes. They are compounds which induce greater order and rigidity in biomacromolecules (Kella and Kinsella 1988). Some, for example, high molecular mass poly(ethyleneglycol)s and ammonium sulphate, are used to precipitate or "salt out" proteins from solution (Wingfield 1998).

Although chaotropicity is a phenomenon familiar to biochemists and biotechnologists, the physical chemistry underpinning it is uncertain and controversial (Ball and Hallsworth 2015; Timson 2019). Early theories focussed on the ability of chaotropes, especially urea, to make strong hydrogen bonds with the peptide backbone of proteins. It was hypothesised that urea successfully competes with intra-molecular hydrogen bonds in proteins and nucleic acids, disrupting tertiary structure (Conway 1956; Von Hippel and Wong 1965). This theory has two major flaws. First, in biomacromolecules, hydrogen bonds are only one of several sources of structural stability (Joh et al. 2008; Pace et al. 2014). While stronger than Van der Waal's forces, a typical biomacromolecule has far more Van der Waal's interactions than hydrogen bonds. The hydrophobic effect also makes a substantial contribution to the thermodynamic stability of proteins and nucleic acids (McMinn et al. 1999; Pace et al. 2011). Steric factors are also important in some cases, notably the DNA double helix (Guckian et al. 2000; Kool et al. 2000). Since many proteins are marginally stable, it could be argued that this does not matter and the breaking of a few (5-10) hydrogen bonds would be enough to begin the unfolding process. However, structural studies on the unfolding of proteins in urea suggest that

some hydrogen bonded structures, such as α-helices, are often among the last to unfold as urea concentrations increase (Fersht et al. 1991; Rocco et al. 2008; Serrano et al. 1992). The second flaw is that some structures disrupted by chaotropes, notably phospholipid membranes, have no hydrogen bonds which contribute to the maintenance of structure.

An alternative explanation for chaotropicity considers the thermodynamics of the system containing solvent (water), biomacromolecule or assembly and chaotrope. Chaotropes typically have high entropies of solution. Following their addition, the entropy of the system rises, mainly due to increased translational and rotational freedom of water molecules. (Thus, the chaotrope's ability to disrupt hydrogen bonding of the *solvent* is more important than its ability to do this to the solute.) Unfolding of proteins or nucleic acids or the disruption of membranes exposes more hydrophobic moieties to the water. This imposes an entropic cost since water molecules form clathrate, or cage-like, hydrogen bonded structures around these hydrophobic groups. The presence of a chaotrope with high entropy of solution can "pay" some of this entropic cost, making unfolding more thermodynamically feasible (Figure 1) (Abu-Hamdiyyah 1965). The reverse argument can be made for kosmotropes (Arakawa and Timasheff 1985). This mechanism does explain the ability of compounds like urea to denature hydrogen bonded and non-hydrogen bonded structures. It is also consistent with the observation that the "strength" of a chaotrope is broadly the same regardless of the molecule or assembly it is added to (Cray et al. 2013). However, it does not explain why some sparingly water soluble, hydrophobic compounds such as benzene can also act as chaotropes.

The lack of a clear, single, molecular mechanism for chaotropicity means that quantitative measurement has proved challenging. Chaotropicity is a phenomenological concept: it is defined by its *effects* on other molecules, not by some fundamental molecular or thermodynamic property (Ball and Hallsworth 2015). This is in contrast to some other parameters commonly measured in biochemistry and biotechnology. For example, pH has an exact definition based on the number of hydrogen ions per unit volume of solvent. It is not defined by the effects of these hydrogen ions on

other molecules. Chaotropicity is more analogous to temperature. Ultimately, temperature is related to the motions of atoms and molecules in the system under investigation. It is measured indirectly by its effects on other molecules, most commonly the expansion of liquids in thermometers. One measure of chao/kosmotropicity uses nanorheology – the measurement of viscoelastic properties on the 1 nm to 1 μm scale (Mukhopadhyay and Granick 2001). This can be used to probe hydration layers around macromolecules in solution. Chaotropes tend to weaken these layers and kosmotropes stiffen them. The extent of this weakening or strengthening could be used to quantify chao/kosmotropicity. To date, this has only been done for fewer than 10 compounds, but the results are broadly consistent with expectations from biochemical experiments (Casanova-Morales et al. 2018). A scale for ions based on solution parameters (ionic radius; water structural entropies; number of surrounding hydrogen bonds) has also been proposed (Assaf and Nau 2018; Marcus 1994; Marcus 2009). This divides ions into kosmotropes (e.g. sulphate and carbonate), chaotropes (e.g. bromide and thiocyanate) and superchaotropes (e.g. ferricyanide, tetrathionate) (Assaf and Nau 2018). This scale, while firmly based on molecular thermodynamics, is only really applicable to ionic species.

The only quantification of chaotropicity which has been applied to the measurement of diverse types of molecules is an empirical one. It relies on the fact that agar gelation is affected by chaotropes (and kosmotropes). Chaotropes increase the gelation temperature and kosmotropes reduce it. By measuring these changes in gelation temperature, Hallsworth and colleagues devised a scale which can be applied from highly chaotropic compounds to highly kosmotropic ones (Cray et al. 2013). It can be used with molecules which are small organics, sparingly soluble in water, polymeric or inorganic compounds as well as with mixtures. Its results are mostly in agreement with experimental realities: for example, guanidinium hydrochloride is highly chaotropic, whereas ammonium sulphate is highly kosmotropic (Cray et al. 2013). This empirical scale reports the molar chaotropicities of compounds (in units of $kJkg^{-1}mol^{-1}$), with positive values being considered chaotropic and negative ones kosmotropic. Experimental evidence suggests that chaotropicities are

not additive (that is, mixing two compounds of chaotropicities X and Y $kJkg^{-1}$ does not generally result in an overall chaotropicity of X+Y $kJkg^{-1}$) (de Lima Alves et al. 2015). Furthermore, it appears that, at least for some compounds, chaotropicity is not a linear function of concentration. (So, if a solution of a given molarity has a chaotropicity of Z $kJkg^{-1}$, it cannot be assumed that a solution of half this concentration has a chaotropicity of 0.5Z $kJkg^{-1}$). A particularly striking example is glycerol which markedly increases its molar chaotropicity above 5 M (Cray et al. 2013).

Inhibition of processes by chaotropicity and kosmotropicity

Since chaotropes disorder biological macromolecules and assemblies, it is not surprising that they are deleterious to cell growth, proliferation and viability. This results from the chaotrope's effects on many targets in the cell, including the cell's membranes, proteins and nucleic acids. All cell types are vulnerable to damage by chaotropes, although some are better able to resist this stress than others. In eukaryotic cells, lower concentrations of chaotropes induce stress responses similar to those which occur in response to elevated temperatures. These include the induction of the unfolded protein response and the production of chaperones which help reverse protein unfolding. In prokaryotes, it has been shown that chaotropes can be used to increase growth at low temperatures (Chin et al. 2010). Presumably, this occurs because the chaotropes partly reverse the low temperature (i.e. lower kinetic energy) rigidification of biomacromolecules. At higher concentrations, chaotropes result in cell death since the stress response systems become overwhelmed, not least since they consist of proteins and nucleic acids which are themselves vulnerable to unfolding. It is important to note that kosmotropes also cause cell stress and, at higher concentrations, death. Biomacromolecules require flexibility to function. It has been proposed that, just as they require a particular three-dimensional structure, they also require an optimum level of mobility (McAuley and Timson 2016). Kosmotropes reduce this mobility and thus may impair function. Many, for example PEGs, also act as macromolecular crowding agents. These

have the effect of reducing the effective volume in aqueous solution thus increasing the effective concentration of enzymes and their substrates (Ellis 2001; Minton 2001; Rivas and Minton 2016). This tends to increase the stability and activity of proteins and, therefore, this crowding effect can act synergistically with kosmotropicity (Wang et al. 2017).

Chaotropic and kosmotropic effects have been demonstrated with isolated biomacromolecules and cellular systems. The unfolding of proteins and consequent loss of activity by urea is well-documented in the literature, as are the effects of milder chaotropes such as ethanol. When the effects of alcohols on the kinetic parameters of β-galactosidase were measured, they tended to decrease the maximum rate (V_{max}) and increase the Michaelis constant (K_m). In the case of ethanol, propanol and butanol (but not methanol), these effects were driven primarily by chaotropicity (Bell et al. 2013). Ethanol (8%v/v) induces the unfolded protein response (UPR) in *Saccharomyces cerevisiae*; the process is triggered by increases in plasma membrane fluidity and it is hypothesised that similar mechanisms may apply in higher eukaryotes (Miyagawa et al. 2014; Navarro-Tapia et al. 2018). The UPR is protective and yeast strains with higher levels of induction of UPR-associated genes are more tolerant to ethanol (Navarro-Tapia et al. 2016). However, in zebrafish, the generation of free radicals in the metabolism of ethanol by cytochrome P_{450} 2E1 is required for maximal UPR induction, suggesting that mechanisms not directly mediated by chaotropicity are also involved (Tsedensodnom et al. 2013). Ethanol and urea inhibit yeast cell growth, reducing the maximum specific growth rate (μ_{max}) and increasing lag time at modest concentrations (2.5-10%). These effects are strain dependent (Brown et al. 1981; Eardley et al. 2019; Hoppe and Hansford 1982; Ranganathan and Bhat 1958).

Kosmotropes such as ammonium sulphate can also inhibit enzyme activity and cell growth (Bell et al. 2013; Nostro et al. 2005). Compared to chaotropes, relatively few studies of the effects of wide concentration ranges of kosmotropes on enzyme activity have been conducted. In some cases kosmotropes activate proteins at lower concentrations (e.g (Darke et al. 1997; Garajová et al. 2017;

Ouyang et al. 2010; Salvucci 1992; Wu et al. 1993)). They also tend to increase the thermal stability of proteins and nucleic acids (e.g. (Barreca et al. 2009; Coelho et al. 2015; de Xammar Oro 2001; Garajová et al. 2017; Mashino and Fridovich 1987; Sampedro and Uribe 2004; Wu et al. 1993)). Both chaotropic and kosmotropic ions inhibit the growth of *Pseudomonas aeruginosa* and *Staphylococcus aureus* above 0.4 M. The magnitude of the effect of a particular ion is different between the two species suggesting a variety of mechanisms, including chao/kosmotropicity, may be operating (Lo Nostro et al. 2006; Nostro et al. 2005). Similar results were observed when the restriction enzyme EcoRI was assayed over a range of ion concentrations (Kim et al. 2001). In the case of enzymes which have kosmotropes as their substrates, inhibition in the physiological concentration range is less likely. For example, the yeast trehalose degrading enzymes Nth1p, Nth2p and Ath1p increase their activity with increasing concentrations of the sugar (App and Holzer 1989; Panek and Souza 1964).

No compound is purely chaotropic or kosmotropic. Compounds can also affect pH, ionic strength, viscosity, crowding, hydrophobicity, water activity, redox potential and osmolarity – all of which can affect the stability and activity of biological macromolecules. This complicates the interpretation of experimental data, even in relatively simple *in vitro* systems. For example, trehalose stabilises proteins (partly due to kosmotropic effects) but can reduce enzyme activity because the increase in viscosity slows diffusion of substrates and domain movements required for catalysis (Sampedro and Uribe 2004). PEG, despite its well-established role as a protein structure stabiliser, causes osmatic stress in plant cells; the latter effect appears to dominate (Chazen and Neumann 1994; Handa et al. 1982; Heyser and Nabors 1981). Some chaotropes and kosmotropes can also act as nutrients, complicating the analysis of cell growth experiments. For example, both urea and ammonium sulphate can act as nitrogen sources; glycerol and trehalose can act as carbon sources.

It is well-established that many processes have optimum temperatures, pH values etc. Given the dramatic effects of chao/kosmotropicity on some biological systems, it is reasonable to postulate that optimal chao/kosmotropicities exist for these processes. Furthermore, since chaotropicity has

some effects in common with increased temperature, particularly elevated mobility of macromolecules and their constituent parts, the prevailing chao/kosmotropicity will affect the optimum temperature.

Industrial fermentations potentially limited by chaotropicity

Some industrial fermentations produce chaotropic substances, most notably alcohols. The production of biofuels, such as ethanol and butanol, is important for energy sustainability and security (das Neves et al. 2007; Paulino de Souza et al. 2018). They can substitute for, or be blended with, liquid fossil fuels. Typically, they are produced by fermentation of plant material by microbes. The species and strains used are often chosen for their resistance to higher alcohol concentrations. The most resistant strains of *S. cerevisiae* cease growing at around 17% ethanol (at 30 °C, but see discussion of sake below) (Duitama et al. 2014; Swinnen et al. 2012). This sets a theoretical upper limit for the amount that can be produced by fermentation. This tolerance is mediated by changes in gene expression which have been mapped. This information can, in theory, be used to inform metabolic engineering of microbes to increase their tolerance to ethanol and other chaotropic substances.

In *S. cerevisiae*, the expression of hundreds of genes is altered in response to moderate ethanol concentrations (5%) (Lewis et al. 2010). These include genes which encode transcription factors, signalling proteins and enzymes involved in lipid biosynthesis. An example of one of these key genes is *ELO1* which encodes fatty acid elongase 1 (EC 2.3.1.199) (Lewis et al. 2010). This enzyme extends fatty acids beyond 14 carbon atoms. Phospholipids which incorporate fatty acids with longer carbon chains are less mobile and, thus, more resistant to increased temperature and to chaotropic agents such as ethanol (Schneiter et al. 2000). Growth at higher concentrations of ethanol, involves other changes in gene expression (Duitama et al. 2014; Swinnen et al. 2012). These include genes which encode enzymes involved in the synthesis of adenine and uracil along with the serine/threonine

kinase Kin3p (Pais et al. 2013). This kinase is involved in the DNA damage response (Moura et al. 2010). Its relevance to ethanol tolerance may be linked so the possible mutagenicity of ethanol at high concentrations (Pais et al. 2013; Ristow et al. 1995). The literature on genetic responses by yeasts to ethanol is considerable and a complete coverage is beyond the scope of this review. However, it is clear that many of the changes reprogram the yeast proteome to deal with stress caused by the chaotropic properties of ethanol – e.g. Increased rigidity of lipids in the cellular membranes, protein chaperones and other systems which promote folding, DNA repair systems, and the production of compatible solutes search as glycerol, trehalose and proline (Cray et al. 2015; Ma and Liu 2010). (The structures of some compounds referred to in this review are shown in Figure 2 and their roles summarised in Table 1.) Similar changes are seen in other species and in the production of other chaotropic substances. For example, the proteome of butanol resistant strains of *Clostridium acetobutylicum* adjusts during the production of butanal so that less mobile, i.e. longer chain and more saturated, fatty acids are produced for incorporation into membrane phospholipids (Mao et al. 2011). Given the common effects on biomacromolecules and cells, likely similar mechanisms, of chaotropes it is reasonable to hypothesise that genomic and proteomic changes which confer resistance to one chaotrope are likely to increase resistance to others.

Vanillin can also be produced by fermentation (dos Santos Barbosa et al. 2008; Muheim and Lerch 1999). However, this compound inhibits the growth of microorganisms in part due to chaotropic mechanisms. Many genetic changes in yeast can result in increased tolerance to vanillin. These include upregulation of the genes encoding the enzymes for ergosterol synthesis in yeast (Endo et al. 2008). Upregulation of the synthesis of this membrane component is also associated with greater tolerance to ethanol (Aguilera et al. 2006). *E. coli* strains which result from artificial selection for higher tolerance to vanillin also show substantial genomic and proteomic changes. Almost 150 proteins have altered levers of expression (Pattrick et al. 2019). Like in yeast, these include proteins associated with stress responses. Chaperone proteins are upregulated along with proteases which

degrade misfolded proteins. Enzymes involved in the synthesis of trehalose are also upregulated and those involved in its degradation are downregulated (Pattrick et al. 2019).

Fewer examples of kosmotropes affecting fermentations have been documented. Ectoine is a kosmotropic compatible solute which is also produced commercially by fermentation (Kunte et al. 2014). It is typically produced by halophilic organisms in media containing high concentrations of sodium chloride. Consistent with its classification as a kosmotrope, ectoine orders water molecules in the bulk solvent (Zaccai et al. 2016). It stabilises, and enhances the activity of, enzymes. However, this effect is limited; at higher concentrations it inhibits the activity (Van-Thuoc et al. 2013). This behaviour is consistent with that of other kosmotropes. It suggests that there will also be a limit to its ability to protect cells and enhance their growth. Furthermore, it is reasonable to hypothesise that the addition of moderate concentrations of chaotropes may enhance the yield of ectoine in fermentations.

Mitigation of chaotropicity

In theory, it should be possible to mitigate the effects of chaotropicity by neutralising the chaotropic agents with kosmotropes. In practice, the situation is more complex. Microorganisms responding to stress often produce compatible solutes. These include proline, betaine, trehalose and glycerol (Brown 1978). With the exception of glycerol, these compounds are kosmotropes (Cray et al. 2013). It is well established that these compounds have a variety of effects within the cell which enable them to survive the various stresses resulting from, for example, ethanol in the surrounding medium. In addition to the production of compatible solutes, some organisms respond to chaotrope stress by altering gene expression. In considering how to mitigate chaotropicity in industrial fermentations, we can draw inspiration from these naturally occurring strategies.

Some organisms can survive high cellular concentrations of urea, e.g. marine elasmobranchs (sharks, skates and rays), holocephalan fish and coelacanths (Yancey and Somero 1979). To counteract its effects, they also produce the kosmotropic compound trimethylamine *N*-oxide (TMAO). This compound has been shown to stabilize proteins in vitro (Qu and Bolen 2003; Yancey and Somero 1979). Molecular dynamics simulations predict that its mechanism of action primarily involves structuring of the water molecules rather than direct interactions with proteins (Bennion and Daggett 2004; Zou et al. 2002). Experimental studies confirmed that the addition of TMAO decreased the entropy of water and this was the main mechanism by which this compound promotes the folding of proteins. Urea had the opposite effect, which could be offset by TMAO (Sahle et al. 2016; Venkatesu et al. 2007; Zou et al. 2002). Neutron scattering experiments demonstrated a direct interaction between urea and TMAO which was optimal at a 2:1 ratio – the same ratio as in elasmobranchs (Meersman et al. 2009; Yancey and Somero 1979). In contrast to earlier work, this suggests that the mechanism of TMAO is not wholly due to interactions with water and relies, in part, on hydrogen bonding between the two molecules (Meersman et al. 2009). Relatively little work has been reported on mixtures of alcohols and TMAO. Molecular dynamics simulations predict, and experiments confirm, that TMAO offsets the chaotropic effects of *tert*-butyl alcohol by a similar mechanism to urea (Di Michele et al. 2004; Fornili et al. 2003).

Since the denaturation of proteins by alcohols occurs partly by a chaotropic mechanism, it is a reasonable hypothesis that TMAO would also partially protect biomacromolecules from high alcohol concentrations during fermentation. This ability to mitigate the effects of chaotropicity appears to be the case for other the kosmotropes and compatible solutes. For example, proline orders water molecules through enhanced hydrogen bonding and protects proteins against denaturation by ethanol (Schobert and Tschesche 1978). Trehalose stabilizes some proteins, prevents denatured proteins from aggregating and helps stablise them in a partially folded form (Singer and Lindquist 1998). Many microbes produce this kosmotrope in response to heat and chaotrope stress, e.g. (An et al. 2011; Odumeru et al. 1993; Sharma 1997; Wang et al. 2014). By preventing aggregation,

trehalose enables denatured proteins to be rescued by chaperones and refolded. However, it also interferes with this refolding process and it is rapidly hydrolized by cells so that it does not do so (Singer and Lindquist 1998). Trehalose also protects yeast cell membranes from disruption by ethanol. This effect is also seen in artificial liposomes with similar lipid compositions suggesting that this effect is a thermodynamic one, not one which requires cellular metabolism (Mansure et al. 1994).

Glycerol's effects are particularly difficult to explain. It is produced as a compatible solute in many species in response to stress. Functionally, it acts in a similar way to the kosmotropes described above. It stablises proteins and phospholipid membranes. However, rather than being a kosmotrope, it can behave chaotropically. Its molar chaotropicity appears to increase as a function of concentration (Cray et al. 2013). This finding is based on the quantification of chaotropicity in the agar gel setting assay. It is not wholly consistent with other experimental findings. Thermodynamic studies on the effects of glycerol on the unfolding of proteins, concluded that it exerts a protective effect partly by ordering the molecules of water – a kosmotropic effect (Gekko 1982; Gekko and Timasheff 1981). Glycerol also causes the compaction of protein structures and prevents aggregation by binding to exposed hydrophobic regions providing an amphipathic interface with water (Vagenende et al. 2009). In contrast, molecular dynamics suggests that glycerol disrupts the hydrogen bonding interactions in water (Chen et al. 2009). Direct interactions between proteins and glycerol may also be important (GhattyVenkataKrishna and Carri 2014). Thermodynamic studies on isolated bases predict that glycerol will destabilize the double helix of DNA (Ganguly and Kundu 1993). Glycerol reduces the enthalpy of denaturation of calf thymus DNA. This is believed to happen because glycerol interacts directly with DNA, replacing hydrating water molecules. Prior to denaturation, there is little change in the conformation of the double helix (Del Vecchio et al. 1999). The effect is larger with GC base pairs compared to AT pairs (Spink et al. 2007). Glycerol does not interact directly with phospholipids in biological membranes. Its effects are indirect and result from ordering of water molecules, both in the bulk phase and close to the membrane (Schrader et al.

2016). These effects are complex and not fully understood. Glycerol can be used to stabilize cell membranes during desiccation or cryopreservation (Keith 1913). In part, this results from glycerol's ability to disrupt the formation of ice crystals (Sieme et al. 2015). Overall, glycerol is hard to classify in the binary system of chaotropes and kosmotropes. It is also a mixed blessing in fermentations. While it acts as a compatible solute to protect cells from ethanol and other alcohols, its production causes problems in downstream processing. Glycerol is miscible with ethanol and, therefore, any glycerol produced or added must be separated from the ethanol adding further costs to bioethanol fermentations.

Sake (Japanese rice wine) is produced by the fermentation of rice husks. Ethanol concentrations up to 20% are possible. Three factors enable this high yield: long fermentation times, low temperature (15 °C) fermentation and selection of highly tolerant yeast strains. Long fermentation times allows the incremental accumulation of ethanol even under non-ideal conditions. The low temperature may partly act by countering the effects of chaotropicity. Adding chaotropes to the medium enabled some species of microorganisms to grow at lower temperatures than have previously been recorded (Chin et al. 2010). It is therefore reasonable to assume that the opposite also holds true - i.e. lower temperatures cause reduced molecular motions (and entropy) and offset some of the deleterious effects of chaotropicity. Of the three approaches used in sake fermentation, only the use of specially selected strains is likely to be commercially viable in biofuel fermentation. The genetic changes seen in sake producing strains result in similar effects to those observed in other strains which ferment to high percentages of ethanol. Genes encoding heat shock proteins along with enzymes involved in trehalose and cell wall biosynthesis are upregulated (Wu et al. 2006). In contrast with many wine and beer making strains, sake yeast have a full complement of genes encoding enzymes for the synthesis of vitamins such as biotin (Wu et al. 2006). Disruption of proline utilization results in the accumulation of this amino acid and greater ethanol tolerance (Takagi et al. 2005).

Although it is routine in industrial fermentations to adjust parameters such as temperature and pH, it is much rarer for the chaotropicity to be deliberately manipulated. Of course, most species used produce compatible solutes as a natural mitigation. The selection of strains for greater ethanol tolerance will often result in the selection of alleles which confer protection against chaotropicity (see above). One straightforward way to mitigate chaotropicity in fermentations would be to "neutralise" it by the addition of kosmotropes. This is not as straight forward as it sounds. No compound is purely kosmotropic and addition of these compounds is likely to affect factors such as the ionic strength, osmolarity, pH or water activity (Lever et al. 2001). In industrial fermentations it is also important to consider the cost of any additional chemicals. Thus, the addition of kosmotropes can only be justified where the cost of the additive is less than the revenue generated by the additional yield. Further complications are suggested by small scale experimental studies. While kosmotropes were able to partially mitigate the effects of urea on yeast growth, they were not as effective against ethanol (Eardley et al. 2019). The reasons for this are not well understood.

Addition of ectoine to cultures of *Zymomonas mobilis* increased the dry mass of the cells at the end of the fermentation, reduced the fermentation time and increased ethanol yield. This occurs partly because ectoine stablises enzyme structures and increases their activity. This effect only occurs up to an ectoine concentration of 1 mM; above this, the compound appears to be inhibitory (Zhang et al. 2008). Addition of glycine, betaine and proline increased the glucose utilisation and cell viability of *S. cerevisiae* in very high gravity fermentations. The mechanism most likely involves a combination of osmoprotection and kosmotropicity (Thomas et al. 1994). The production of glycerol by fermentations is not always desirable. Engineering a yeast strain which had reduced glycerol production resulted in reduced tolerance to osmotic stress. However, overexpressing the enzymes of the trehelose synthesis pathway reversed this effect and resulted in higher ethanol yields than observed in the wild type strain (Guo et al. 2011). This demonstrates that the replacement of a less desirable compatible solute with a more desirable one can result in yield improvements and potential cost saving benefits. Overexpression of the genes which encode the enzymes for proline

biosynthesis and for the uptake of its precursor, glutamate, enhanced the butanol tolerance of *Bacillus subtilis* 168 - a strain which naturally produces butanol (Mahipant et al. 2017). Taken together, these diverse studies strongly suggest that the supplementation of fermentations with appropriate, kosmotropic compatible solutes is likely to enhance productivity.

A number of factors will need to be considered when choosing the most appropriate kosmotrope. While cheap, and widely available, glycerol is hard to remove from liquid products with which it is miscible, for example alcohols. Ammonium sulphate is also cheap and widely available. However, high concentrations can inhibit growth (Eardley et al. 2019). The "strength" of the kosmotrope is also an important consideration. Some cheap kosmotropes, for example sucrose, have relatively low molar kosmotropicities (Cray et al. 2013). A recent analysis of the cost per unit kosmotropicity concluded that polyethylene glycols may be the best choice on economic grounds (Timson and Eardley 2020). However, these compounds are unlikely to enter the cell and significant concentrations are likely to result in osmotic stress on the microbial cells.

Unanswered questions

This review has focused largely on the production of ethanol by the yeast *S. cerevisiae*. The universality of the effects of chaotropes on cells mean that many of the conclusions should be generalizable to other fermentations, to the production of other substances and other species. Nevertheless, there are unanswered questions and research needs. Perhaps the most important is a method for the rapid measurement or accurate prediction of chaotropicity in complex mixtures. This would mean that additions could be made to fermentations which would result in controlled, predictable alterations to the chaotropicity of the mix (Figure 3). To achieve this, a robust device which could repeatedly and accurately measure net chaotropicity or a rigorous mathematical model which would enable its calculation is required. The former requires a novel method for the empirical measurement of chaotropicity. While the agar gel melting assay is applicable to a wide range of

compounds, it is not reusable in its current form. Any method used industrially would need to be capable of making hundreds of accurate measurements without servicing, repair or replacement of key parts and reagents. Ideally it would be suited to in-line measurement so as to enable automated monitoring, feedback and correction. The latter is currently impossible due to the lack of a complete physical understanding of the phenomenon. Given the phenomenological nature of chaotropicity, this may not be possible. However, if it was, it would enable the rapid estimation of net chaotropicity in a similar manner so the estimation of pH using the Henderson-Hasselbalch equation. Both an empirical system and quantitative estimation, would fail when dealing with glycerol. This compound's behaviour is not well understood and it may defy simple classification.

In conclusion, chaotropicity can be recognised as one of many factors which affect the success of fermentations and the yield of products. Ideally its mediation should be simple. However, the reality is more complex. The current state of knowledge does not permit the rational design of fermentations with the optimal chaotropicity. Nevertheless, an appreciation of its role in limiting growth and yields, will permit empirical, trial and error methods to be adopted. Further experimental work, including small scale laboratory studies, is required to determine the most effective means to neutralise excess chaotropicity.

Conflict of interest statement

The author has no conflicts of interest to declare.

Acknowledgements

The author thanks students and colleagues who are providing many stimulating discussions on this topic over many years.

Figure legends

Figure 1: A schematic diagram to illustrate the thermodynamic explanation of chaotropicity. Only entropy is considered. It is assumed that enthalpy will be unchanged in the presence of the chaotrope. This is most likely an oversimplification, especially if the chaotrope also interacts directly with the protein. In the absence of chaotrope, there are two major, opposing entropy changes. The unfolding of the protein results in greater conformational freedom and a positive entropy change ($\Delta S_{flexibility}$). However, the consequent exposure of hydrophobic regions requires the ordering of water molecules around these regions which reduces the translational freedom of these water molecules and thus results in a negative entropy change ($\Delta S_{solvhydr}$). Overall, these changes are often similar in magnitude and the net entropy of unfolding ($\Delta S_{unfolding}$) is close to zero. The presence of a chaotrope in the solution introduces a third entropy change associated with dissolving this compound ($\Delta S_{disschao}$). This arises from two main factors: the dissolving of the solute into the water which disperses they solute molecules and the disruption of hydrogen bonding networks in the water which results in greater translational freedom for the water molecules. Therefore, the overall entropy of unfolding in the presence of a chaotrope is likely to be larger and more positive. The analogous, reverse argument applies to kosmotropicity.

Figure 2: Structures of some of the compounds mentioned in this review.

Figure 3: Methods to mitigate chaotropicity in industrial fermentations. (a) A chaotropicity sensor is located in the fermentation mix constantly measuring they net chaotropicity. It provides feedback to a kosmotrope reservoir, controlling addition to enable constant adjustment of the net chaotropicity. (b) Alternatively, the chaotropicity could be calculated from the results of analysis of the composition of the fermentation mix. The amount of kosmotrope to be added would then be

calculated. Note that neither chaotropicity sensors nor robust methods for calculating net chaotropicity exist. Furthermore, the optimum chaotropicity for a particular fermentation remains to be determined.

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Tables

Table 1: The main chaotropes and kosmotropes covered in this review.

Compound	Molar chaotropicity[a]/ $kJkg^{-1}mol^{-1}$	Roles in fermentation	References
Ammonium sulphate	-66.9	Precipitates proteins; inhibits cell growth and enzyme activity at high concentrations	(Bell et al. 2013; Eardley et al. 2019; Wingfield 1998)
Betaine	-25.5	Compatible solute produced by some microorganisms in response to chaotrope and other stresses	(Arakawa and Timasheff 1985)
Butanol	+37.4	Chaotropic fermentation product which will limit its own production	(Cray et al. 2015)
Ectoine	-16.6	Kosmotropic fermentation product which may limit its own production; Possible additive in chaotrope producing fermentations	(Kunte et al. 2014; Zhang et al. 2008)
Ethanol	+5.9	Chaotropic fermentation product which will limit its own production; most widely manufactured biofuel.	(Cray et al. 2015; das Neves et al. 2007; Paulino de Souza et al. 2018)
Glycerol	+6.1 (6.5-10M)/+1.1 (<5M)	Compatible solute produced in response to multiple stresses; paradoxically it is mildly chaotropic	(Bhaganna et al. 2016; Cray et al. 2013; Gekko and Timasheff 1981; Hallsworth et al. 2003; Nevoigt and Stahl 1997)
Polyethylene glycol	Kosmotropic, but depends on molecular mass	Potential additive to mitigate chaotropicity in fermentations	(Timson and Eardley 2020)
Proline	-5.8	Compatible solutes produced by some microorganisms; mitigates chaotropicity and other stresses.	(Takagi 2008)
***tert*-Butyl alcohol**	nd	Probable chaotrope which can be “neutralised” by TMAO.	(Di Michele et al. 2004; Fornili et al. 2003)

Trehalose	-10.6	Compatible solute produced by some microorganisms in response to chaotrope and other stresses	(Mansure et al. 1994; Swan and Watson 1998; Wang et al. 2014)
Trimethylamine *N*-oxide (TMAO)	-25.9	Kosmotrope used by some marine organisms to mitigate the chaotropic effects of urea; Possible additive in chaotrope producing fermentations	(Arakawa and Timasheff 1985; Bennion and Daggett 2004; Yancey and Somero 1979)
Urea	+16.6	Widely used for the denaturation of proteins in laboratory studies; model compound in many offset experiments	(Ahmad and Bigelow 1982; Bennion and Daggett 2003; Das and Mukhopadhyay 2009; Zou et al. 2002)
Vanillin	+174	Chaotropic fermentation product; by-product of pre-processing steps for some biofuel fermentation substrates.	(Cray et al. 2015; Li et al. 2014; Oliva-Taravilla et al. 2015; Pattrick et al. 2019)

[a] Values from (Cray et al. 2013). Positive values represent chaotropes and negative values kosmotropes; nd, not determined

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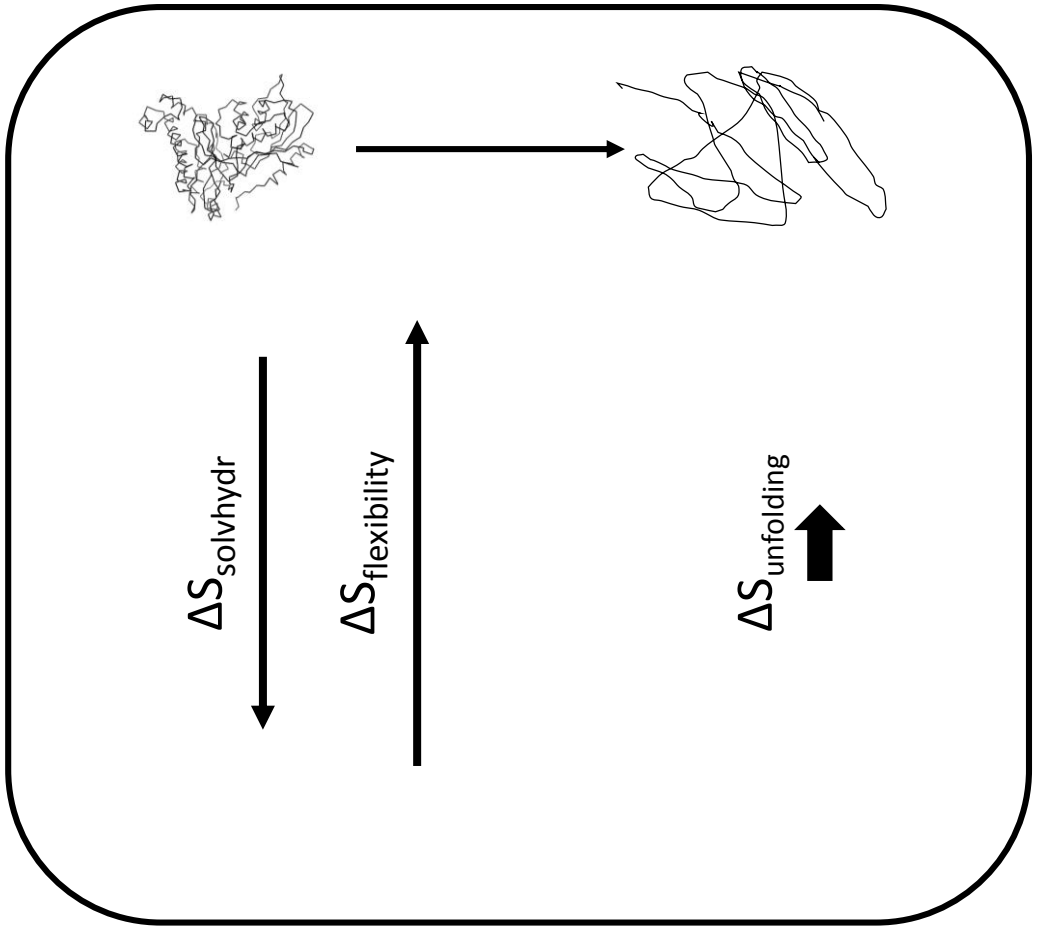
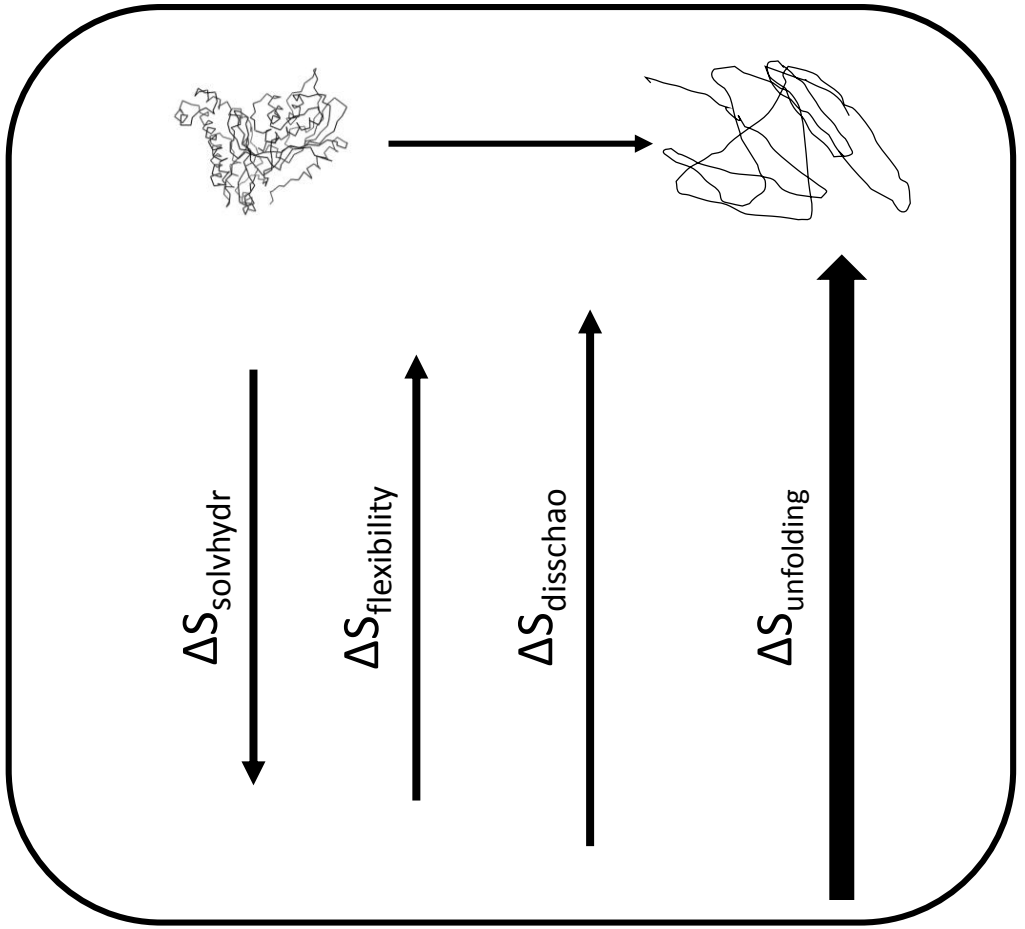


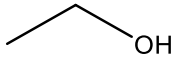
Protein unfolding in water



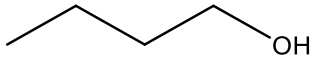
Protein unfolding in water plus chaotrope

fig 2

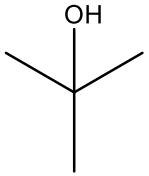
Click here to access/download;Figure;fig2.pptx



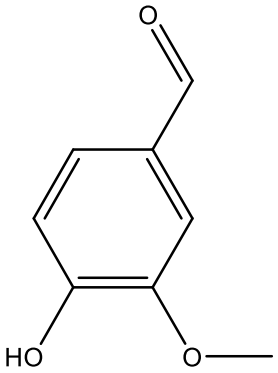
Ethanol



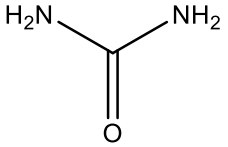
Butanol



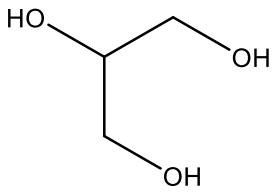
Tert-butyl alcohol



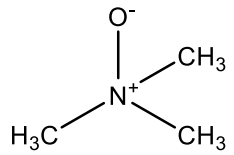
Vanillin



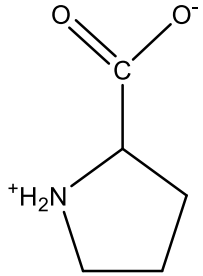
Urea



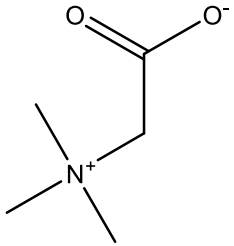
Glycerol



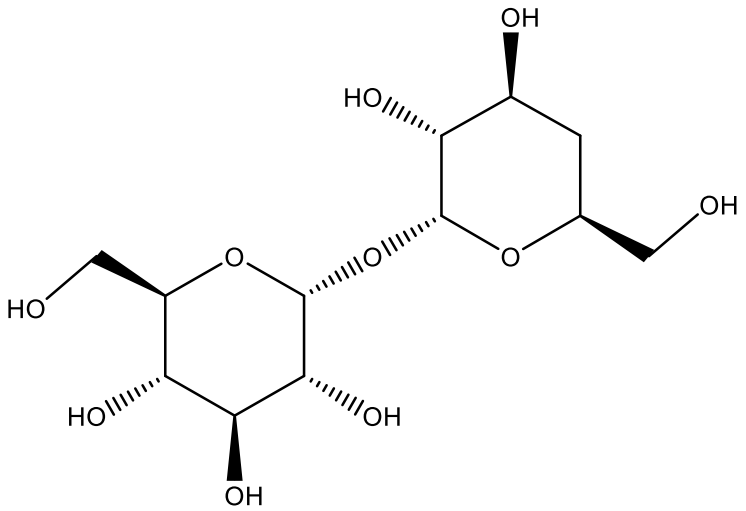
Trimethylamine *N*-oxide (TMAO)



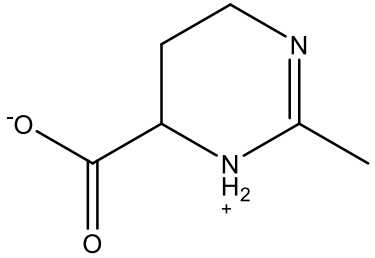
Proline



Betaine

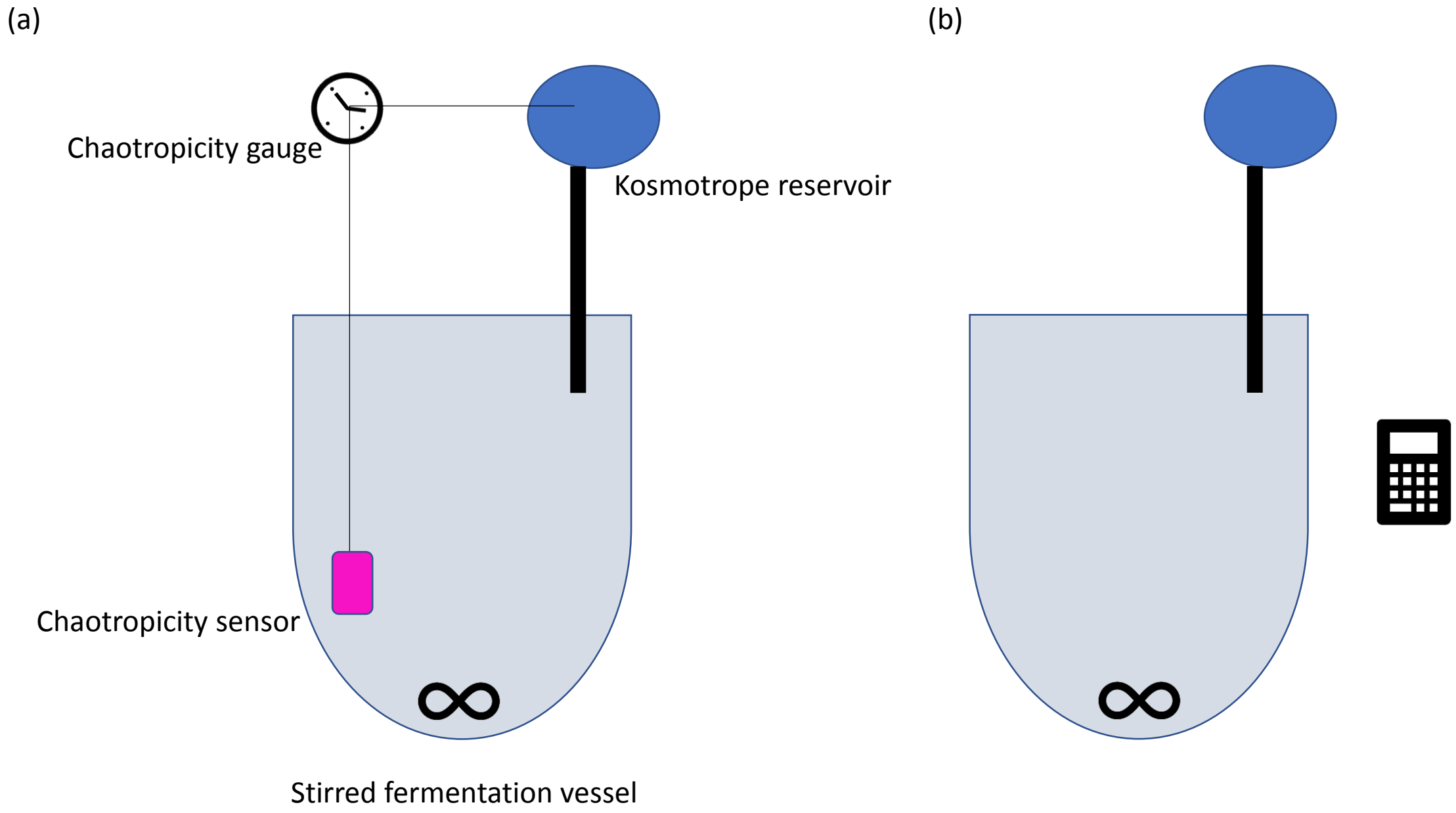


Trehalose



Ectoine

Figure 3



checklist

cover letter