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REVIEW

How do yeast cells become tolerant to high ethanol concentrations?

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Abstract The brewer's yeast *Saccharomyces cerevisiae* displays a much higher ethanol tolerance compared to most other organisms, and it is therefore commonly used for the industrial production of bioethanol and alcoholic beverages. However, the genetic determinants underlying this yeast's exceptional ethanol tolerance have proven difficult to elucidate. In this perspective, we discuss how different types of experiments have contributed to our understanding of the toxic effects of ethanol and the mechanisms and complex genetics underlying ethanol tolerance. In a second part, we summarize the different routes and challenges involved in obtaining superior industrial yeasts with improved ethanol tolerance.

Keywords *Saccharomyces cerevisiae* · Ethanol tolerance · Fermentation · Biofuel

Saccharomyces cerevisiae produces and withstands high ethanol titers

Tolerance to (high) ethanol levels is a key characteristic of the yeast *Saccharomyces cerevisiae* (Stanley et al. 2010). One obvious reason why yeast cells have evolved the capacity to withstand high levels of ethanol is because they actually produce this toxic molecule. Even under aerobic conditions, *S. cerevisiae* prefers to metabolize sugars through alcoholic fermentation rather than respiration—the so-called Crabtree effect (Crabtree 1928; Pronk et al. 1996). It is widely believed that this preference for fermentation is explained by the faster production of ATP during fermentation compared to respiration, by the growth inhibitory effect of ethanol on competing microbes that are not as ethanol tolerant, and by the ability of *S. cerevisiae* cells to respire the ethanol they produced after the levels of fermentable sugars have dropped (Piskur et al. 2006; Thomson et al. 2005).

Ethanol tolerance has received much scientific attention in part because many industrial applications such as the production of alcoholic beverages or fuel alcohol rely on *S. cerevisiae*. In the last stages of these processes, cells are confronted with ethanol concentrations that are so high that they also become toxic to yeast cells. A further increase in ethanol tolerance in these industrial yeasts could result in faster and more complete fermentations, and may also allow the production of liquids that contain even more alcohol, which would increase production yields, efficiency and sustainability. Interestingly, different yeast strains can show very different abilities to grow and/or survive in the presence of ethanol. This suggests that some yeast strains harbor certain alleles that allow these strains to better fight the toxic effects of ethanol (Mukherjee et al. 2014; Steensels and Verstrepen 2014). In this perspective, we discuss different approaches used to identify the genetic determinants of ethanol tolerance.

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Approaches used to unravel determinants of ethanol tolerance

Because of the biological, ecological and industrial importance of ethanol tolerance, several studies have set out to investigate why ethanol is toxic to cells, and to identify the molecular mechanisms underlying tolerance to ethanol.

A first obvious strategy involves the use of genome-wide screens to investigate the effect of individual gene deletions on ethanol tolerance. Multiple screenings of single gene knock-outs as well as transcriptomics studies of ethanol-stressed cells indicated that genes influencing cell membrane composition and protein folding are generally important for ethanol tolerance (Avrahami-Moyal et al. 2012; Inoue et al. 2000; Sanchez et al. 1992; Swinnen et al. 2012; van Voorst et al. 2006; You et al. 2003) (see also Table 1). Moreover, results from these studies also suggested that the mechanism of ethanol tolerance is concentration dependent, with different genes contributing to tolerance to low and high ethanol concentrations, respectively (Alexandre et al. 2001; Fujita et al. 2006; Lewis et al. 2014; van Voorst et al. 2006). In other words, these results indicate that some mechanisms might be highly relevant for handling a lethal concentration of ethanol, but are less relevant for sublethal ethanol levels, and vice versa. Moreover, in the past years, it has become increasingly clear that ethanol tolerance is a complex, polygenic phenotype that can be influenced by multiple alleles that may show complex interactions and that may differ between different strains.

Whereas genome-wide screens that investigate the effect of gene deletions are useful to identify key physiological processes contributing to ethanol tolerance, they do not allow determining which genetic variations are responsible for the large differences in ethanol tolerance between different yeast strains. More recent studies have therefore used a quantitative trait loci (QTL) mapping strategy to identify genes underlying ethanol tolerance in laboratory strains, as well as in commonly used industrial strains (Ehrenreich et al. 2010; Hu et al. 2007; Swinnen et al. 2012). For example, a pooled-segregant whole-genome sequencing QTL strategy identified several alleles, including specific alleles of Vps70 and the chaperone Apj1, underlying tolerance of a Brazilian bio-ethanol strain to high ethanol levels (Duitama et al. 2014; Swinnen et al. 2012) (see also Table 1).

Researchers have also looked at adaptation to ethanol in originally non-ethanol tolerant microbes exposed to gradually increasing levels of ethanol (Avrahami-Moyal et al. 2012; Stanley et al. 2009; Voordeckers et al. 2015). These studies have shed light on the physiological adaptations found in evolved, more ethanol tolerant cells. In line with findings from large-scale deletion screens, cell wall stability was found to be one of the major phenotypes that changed during adaptation to ethanol (Avrahami-Moyal et al. 2012). Our group recently performed an extensive analysis of the mechanisms and genetic changes underlying long-term adaptation of independent yeast populations to gradually increasing ethanol levels (Voordeckers et al. 2015). Although we found little overlap between the genes mutated in the different evolved lineages, several functional modules are affected in multiple adapted populations; including pathways involved in stress response, intracellular signal transduction, cell cycle and membrane composition and organization.

Taken together, studies have linked over 200 genes to ethanol tolerance (see also "Challenges") and identified many different cellular processes affected by ethanol, such as membrane fluidity and composition, as well as activity and folding of proteins (see also Table 1) (Ding et al. 2009; Ma and Liu 2010; Stanley et al. 2010). The cell membrane is a key target of ethanol because ethanol increases membrane fluidity, disrupts membrane structure and can inhibit membrane proteins directly. Several studies indicate that

Table 1 Genes and processes associated with ethanol tolerance

Cellular function	Gene(s)	Reference(s)
Cell membrane composition/cell wall stability	*OLE1*, *ERG6*, *SSD1*, *UTH1*	Avrahami-Moyal et al. (2012), Inoue et al. (2000), van Voorst et al. (2006) and You et al. (2003)
Electrochemical gradient	*PMA1*, *TRK1*	Alper et al. (2006) and Rosa and Sa-Correia (1991)
Vacuole/endocytosis	*VPS70*, *VPS16*, *VPS28*, *VPS34*, *VPS36*, *MEH1*	Duitama et al. (2014), Takahashi et al. (2001), van Voorst et al. (2006) and Voordeckers et al. (2015)
Trehalose	*TPS1*, *ATH1*	Kim et al. (1996) and van Voorst et al. (2006)
Heat shock proteins	*HSP104*, *APJ1*	Sanchez et al. (1992) and Swinnen et al. (2012)
DNA damage repair and chromosome integrity	*PAT1*, *ADE1*, *KIN3*	Pais et al. (2013) and Takahashi et al. (2001)
Amino acids and nutrient uptake	*TAT1*, *BAP3*, *LYS2*, *LEU2*, *HIS3*, *MET15*, *TAT2*	Baerends et al. (2009), Hirasawa et al. (2007) and Swinnen et al. (2015)

the level of unsaturated fatty acids as well as the level of ergosterol in yeast cell membranes affects ethanol tolerance (Henderson and Block 2014; Vanegas et al. 2012). Accumulation of ethanol in the cell membrane has been suggested to lead to ion leakage and dissipation of the electrochemical gradient, thereby inhibiting the uptake of nutrients that depend on this gradient for their transport over the membrane (Cartwright et al. 1987; Madeira et al. 2010). In addition, ethanol likely also affects general protein folding and stability (Ma and Liu 2010).

Improving ethanol tolerance

Given the industrial importance of ethanol tolerance, several attempts have been made to obtain superior yeast variants or mutants. Examples include

- Introduction of specific mutated alleles, identified in an experimental evolution study, into a non-ethanol tolerant strain significantly increased ethanol tolerance (Voordeckers et al. 2015). This demonstrates the adaptive nature of these mutations as well as the potential of experimental evolution approaches to unravel and improve a complex phenotype. Notably, the effect of these mutations depended on the ethanol concentration. This again indicates that the mechanisms underlying tolerance to low and high ethanol levels are different. Introduction of these mutations in an industrially used, diploid bio-ethanol strain also enhanced its ethanol tolerance (Verstrepen et al. 2015). These findings show that, although polygenic, alcohol tolerance can significantly be improved by altering a single process.
- Given the complex nature of ethanol tolerance, it seems logical that a large increase in ethanol tolerance requires several changes in the yeast's genome. Several approaches that rely on the effect of (random) variation generated by (genome-wide) mutagenesis or genome shuffling have successfully yielded strains with increased ethanol tolerance (Alper et al. 2006; Snoek et al. 2015). Snoek et al. have, for example, obtained superior strains through large-scale robot-assisted genome shuffling experiments that allowed to combine beneficial alleles of different highly tolerant natural strains into new hybrid backgrounds (Snoek et al. 2015). In a different approach, Alper and colleagues created strains with improved ethanol tolerance by global transcription machinery engineering. They created a mutant library of the TATA binding protein Spt1, and transformed this library into an auxotrophic laboratory yeast strain followed by plating on medium containing high levels of glucose and ethanol. Mutation of this global transcription factor led to wide-spread transcriptional reprogramming and several mutant strains displayed increased viability in ethanol. Upregulated genes in these mutants were enriched for functional groups such as cytoplasmic proteins and amino acid metabolism. Moreover, each of the most upregulated genes was found to be essential for the increase in ethanol tolerance. However, the exact molecular details and mutations resulting in increased ethanol tolerance were not identified. Interestingly, a later study demonstrated that the phenotypic improvement of one of the Spt1 mutants was caused by fixing a defect in leucine uptake/utilization of the auxotrophic laboratory strain, and could only be reproduced in medium with low amounts of leucine (Baerends et al. 2009) (see also "Challenges").
- A very different approach was taken by Lam and coworkers, who used knowledge of the toxic effect of ethanol to change the external growth medium in such a way that cells can better cope with high ethanol concentrations. Specifically, they showed how the ion composition of the growth medium can significantly enhance ethanol tolerance (Lam et al. 2014). Similar results were obtained by genetically engineering genes affecting the K^+ importer and H^+ exporter levels. Increased concentrations of K^+ as well as increased pH of the extracellular medium decreases ion leakage caused by ethanol, by reducing intra- and extracellular concentration differences. Interestingly, this approach increased ethanol tolerance independent of strain background.

Challenges

Apart from the complex genetics involved, several other factors complicate research into ethanol tolerance, which further explains why the results of many studies show little overlap (Stanley et al. 2010). A key confounding factor is the lack of a standardized vocabulary. Although ethanol tolerance is an extremely relevant phenotype, there is no widely accepted definition, nor a standardized method to assay this phenotype. The most widely used definition of ethanol tolerance is the ability to grow (undergo cell division) in the presence of ethanol (Casey and Ingledew 1986; Swinnen et al. 2012; Voordeckers et al. 2015), although some studies define ethanol tolerance as the degree of survival after exposure to ethanol (Hu et al. 2007) or as the maximal ethanol production capacity (Pais et al. 2013). Moreover, the use of different experimental conditions (ethanol concentration, time of incubation, composition of medium, strain background) further complicates the direct comparison of different studies. Lastly, ethanol is a highly volatile substance that is produced during normal cell growth. This implies that the concentration of ethanol

in the actual experiments can be very different from what is reported, since the reported concentration is often based on the concentration that was added to the medium or buffer.

Another important caveat of many (large-scale) studies investigating ethanol tolerance is that they are often performed with (an auxotrophic version of) the laboratory yeast strain S288c. This strain was the first eukaryote of which the whole-genome sequence was determined (Goffeau et al. 1996), which in turn fueled the development of a large set of molecular tools for this strain, such as gene knock out collections. These tools have proven to be extremely useful to better understand a multitude of phenotypes, including ethanol tolerance. However, recent studies indicate that S288c, and in particular its auxotrophic version, often performs very different from prototrophic and/or natural strains (Mulleder et al. 2012; Pronk 2002; Warringer et al. 2011; Wohlbach et al. 2014). The results obtained by Alper and co-workers, for example, have to our knowledge not been reproduced in industrial strains (Alper et al. 2006; Baerends et al. 2009). Moreover, Pais and co-workers found that an auxotrophic version of S288c displays lower ethanol tolerance and production compared to natural and industrial strains (Pais et al. 2013). Interestingly, auxotrophic mutations have recently been found to significantly reduce tolerance to high levels of ethanol (Swinnen et al. 2015). Since strains carrying these auxotrophic mutations cannot synthesize certain building blocks, they rely on import of these nutrients for their growth and survival and it is exactly this import that is affected by ethanol. To overcome the problems associated with these auxotrophies, several groups have created a prototrophic version of the yeast deletion collection (Mulleder et al. 2012; VanderSluis et al. 2014), but a detailed characterization of these collections for ethanol tolerance is still lacking.

Conclusion

It is becoming increasingly clear that ethanol resistance is an extremely complex phenotype. Firstly, ethanol tolerance involves multiple physiological processes, that each rely on many different genes. Moreover, it seems that many different combinations of alleles and mutations can lead to improved ethanol tolerance with complex interactions between these mutations. Alleles that increase fitness in moderate ethanol concentrations do not necessarily also have a positive effect in high ethanol concentrations, and vice versa. A lack of standardized definitions of ethanol tolerance further complicates interpretation and comparison of results.

Despite these confounding factors, however, much progress is being made. A bird's eye view of the results obtained so far clearly identifies a few key physiological processes that seem to determine a cell's fitness in high ethanol concentrations (see Table 1). Moreover, altering only one of these pathways can already significantly affect ethanol tolerance, opening up new routes for rational strain improvement. The most significant increases in ethanol tolerance, however, can be expected when beneficial alleles are combined. Given the multitude of identified alleles, and the complex genetic interactions that likely exist between these alleles (and also the rest of the genome), this still a daunting, but exciting, challenge.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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