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Review paper

Multi-Stress Response Mechanism of *Kluyveromyces marixanus* and Techniques to Improve Stress Tolerance

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Abstract

During the ethanol fermentation process, yeast cells are subjected to a variety of stressors, including excessive osmolarity caused by sugar substrates, raised ethanol levels, the production of reactive oxygen species (ROS) from oxygen consumption, and rising temperatures. To effectively manage the problems associated with fermentation, suitable adaptive responses must be activated. These processes are essential to protect cells from stress-induced loss and to acquire the ability to resist stressful conditions. This review aims the cellular repercussions of different stresses, the cellular mechanisms that contribute to stress tolerance and techniques to improve stress tolerance. Given that a single stressor has the potential to cause a wide range of impacts, including both specific and non-specific outcomes, a comprehensive defence strategy requires both specialised and generalised stress responses. Because of the shared influence of these stresses on protein structural disruption, when yeast cells are subjected to any of these stressors, there is a common induction of higher amounts of heat shock proteins (HSPs) and trehalose. As a result, acquiring a deeper understanding of the mechanism behind yeast tolerance to certain stresses inherent in fermentation is critical, especially when it comes to improving yeast stress tolerance using different strain engineering procedures.

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INTRODUCTION

To address the growing need for energy and the adverse environmental implications associated with fossil fuels, many countries have seen an increase in the use of ethanol as an environmentally safe alternative fuel. During fermentation for ethanol, yeast cells are confronted with a variety of stresses, which include high sugar stress (osmotic stress), end-product inhibition (ethanol stress), thermal stress, and reactive oxygen species (ROS; oxidative stresses), which are all imposed concurrently and sequentially [1]. The beginning of fermentation when these are hurled into a fermentation inoculum yeast cells first experience osmotic stress because of the high quantity of sugar substrates especially when high gravity ethanol fermentation is carried out (> 20/22% w/v) [2]. On the contrary, as the concentration of ethanol increases, stress become critical near the ending of the fermentation process, which also inhibits yeast development and metabolism and results in ethanol production termination when concentration reached >7% in *K. marxianus* [3]. Both the stress affects *K. marxianus* cells adversely and results in damage to yeast cells. Ethanol

fermentation is mainly semi-anaerobic; oxygen is one of the important factors for the growth of culture before they are added to scaleup level. Moreover, ROS and other oxygen derivatives are formed when oxygen levels in the electron transport chain are reduced. In fermentation on an industrial level, the temperature is normally managed by a water-cooling system and due to high external temperatures, this is especially common in areas with high ambient temperatures, such as during the summer or in tropical countries like India. As a result, increased temperatures pose an additional difficulty to *K. marxianus* cells during fermentation. To manage these fermentation-related stressors, *K. marxianus* cells must activate appropriate biological responses. These reactions are critical for yeast cells in terms of preventing from stress-induced damage and establishing resilience to difficult conditions. Understanding the cellular mechanisms by which yeast responds to the numerous challenges faced during fermentation is critical [4]. This understanding is essential for the effective generation of engineered yeast strains with greater tolerance to numerous stresses, a highly desirable characteristic for optimising bioethanol yield. As a result, this review will emphasis on the present level of knowledge about the cellular processes governing yeast responses and resilience to the impact of ethanol fermentation stressors and different techniques for improving stress tolerance to yeast.

Multi stress conditions

Understanding the key basis of *K. marxianus* responses to diverse stresses encountered during fermentation is critical for the effective development of genetically engineered, multi-stress tolerant yeast strains that should have preferred characteristics for economical ethanol production from *K. marxianus* cells (Fig.1.).

Thermal stress

Increased fermentation temperature beyond an acceptable range during commercial ethanol fermentation can impact yeast metabolism and survivability, leading to a fall in ethanol yield. Thermal stress causes protein malfunction, metabolic abnormalities, and cellular disintegration by disrupting the structure and function of proteins, enzymes, cell plasma membranes, and internal cytoskeleton structures [5]. The response against thermal stress is first to avoid protein aggregation by producing HSPs, and other protective solutes of yeast cell i.e., trehalose, repair of cell membrane shown in Fig 1.

HSPs expression mediated by heat shock factor 1 (Hsf1) and zinc finger protein (Msn2/Msn4). These transcription factors are important in the thermal stress response [6]. HSF1 regulates the expression of genes that are targeted, particularly those involved in degradation and protein folding, ATP production, sugar utilization and maintaining cell membrane structure. When activation of HSF1 occurs, it binds to it binds to heat shock element motif in promoter site. Whenever, thermal shock faced by yeast cell, HSF1 hyperphosphorylated which positively regulated by C-terminal regulatory domain [7]. MSN2/MSN4 is general stress response transcription factor, which regulate genes transcription in multi-stress condition that includes thermal, ethanol, oxidative, osmotic by binding through stress response elements 8 (Watanabe *et al.,* 2009). Under stressful conditions, MSN2/MSN4 undergo hyperphosphorylation and subsequent translocation into the nucleus. This nuclear entry and exit of MSN2/MSN4 are regulated by the cAMP-dependent protein kinase pathway and occur periodically at specific time intervals [9].

Heat Shock Proteins are a type of molecular chaperone that aids in the folding of molecules and refolding of proteins which are newly synthesize and misfolded proteins respectively. HSPs also disaggregate, aggregated proteins under stress. HSPs are quality control agent of protein in cell under stressed and non-stressed conditions. These were classified according to their weight into small HSPs (<50kda) and large HSPs (>50kda). Unlike HSP70 chaperones, generally identify misfolded or unfolded proteins without discrimination. The activity of the HSP90 protein is critical for the folding of certain target molecule of proteins, such transcription factors that control gene regulations and enzyme kinases. Role of small HSPs is to bind unfolded proteins that inhibit irreversible aggregation of proteins. Under stress condition HSP26, HSP42 which exist as part of a big homo-oligomeric complex form, breakdown into dimeric by interacting with non-folded proteins [10]. Trehalose is a disaccharide sugar that play role as a protectant of protein, being denatured, and aggregated during heat stress, also stabilize structure of protein by binding to non-folded to maintain their integrity in partially folded state. Gene trehalose-6-phosphate synthase (TPS1), and trehalose-6-phosphate phosphatase (TPS2) of trehalose upregulate under stress that results in increase in concentration intracellularly [11].

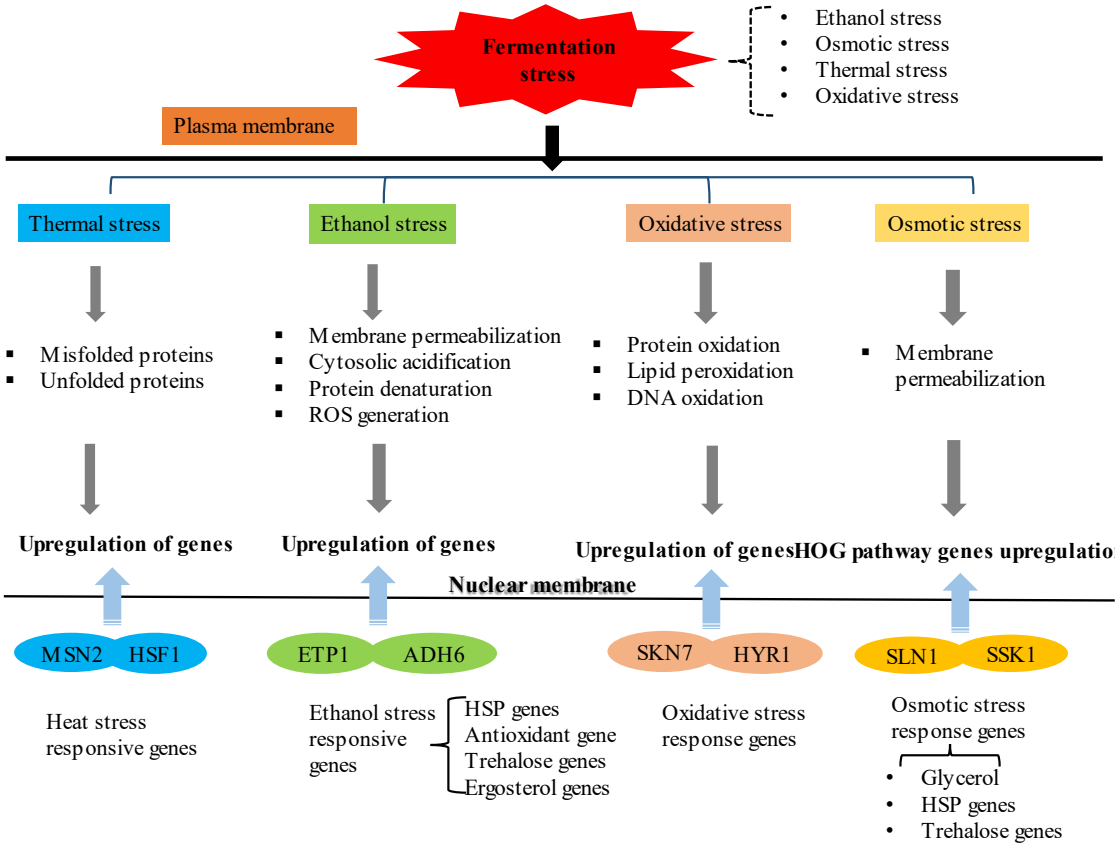

Fig 1: Stress response by *K. marxianus* under multi-stress condition

Ethanol stress

Ethanol is a major metabolite produce during fermentation, which represents another substantial stress that *K. marxianus* cell faces. As the concentration of ethanol increase in medium, viability of yeast cell reduces, which leads to termination of fermentation. Ethanol causes cellular toxicity by inhibiting sugar and amino acid uptake, lowering activity of glycolytic enzymes, as well as membrane integrity [12].

Ethanol targets mainly plasma membrane by intercalating into interior hydrophilic lipid bilayer, that leads to loss in integrity and permeability of membrane. The increase in permeability of membrane increases influx of ion, mainly protons, that triggered cytosolic acidification. the vacuole H+-ATPase and plasma membrane H+-ATPase systems are involved in moving protons into the vacuole and sending them out of cells in reaction to variations in influx [13]. To cope up the membrane disrupting effect of ethanol, *K. marxianus* changes components UFAs and ergosterol are components of the plasma membrane that aid in keeping membrane shape and fluidity. The major UFAs that change in concentration are palmitoleic and oleic acids. You *et al.*, (2003) [14] reported that transformants of ethanol tolerant produce more oleic acid compare to palmitoleic acid, when there was no ethanol however, it produces 4-fold higher oleic acid under 5% ethanol stress. Under the exposure of ethanol, many intracellular components such as protein, glycolytic enzymes disrupt. To prevent these effects of ethanol yeast cell, express several genes which are associated with HSPs and trehalose that become up-regulated after exposure.

HSP genes for e.g., HSP26, HSP78, HSP104, HSP12 are upregulate during exposure and prevent denaturation of protein by refolding of miss-folded protein and prevents or disassemble protein aggregation [15]. Trehalose plays similar role as HSPs play to prevent protein denaturation, it plays close interplay by binding with protein and replacing water surrounding protein that maintains water activity [12]. Several amino acids such as proline, tryptophan, and arginine aids in protecting yeast cell under ethanol stress in addition to the HSPs and trehalose (Fig.1). Amino acid arginine protected yeast cells under damaging stress of ethanol by maintaining the structure of the cell wall and internal cytoplasmic membrane, as well as maintaining organelle structure and function due to reduced ROS production [16].

Oxidative stress

The situation of oxidative stress is created by an internal pro-oxidant/antioxidant imbalance that favours the pro-oxidants. ROS are the most common internal pro-oxidants and are oxygen-derived molecules with one or more unpaired electrons. The production of ROS occurs spontaneously, in result of environmental shocks and by-products of regular aerobic metabolism. Through the process of oxidative phosphorylation, mitochondrial respiration is assumed to be the principal generator of ROS in cells. Superoxide anion (O_2^-), hydrogen

peroxide (H_2O_2), and hydroxyl radical (˙OH) are the major cellular ROS [17]. Oxidative damage due to accumulation of ROS results in damage of cell components such as DNA, lipid, and protein that leads to cell death. Protein damage due to ROS affects cell's homeostatic function and reduce viability of cell. This also associates with cell aging and accelerate free metal ions. Major yeast protein targets are citric acid cycle enzymes which inactivate by H_2O_2 and superoxide radicles. DNA damage due to oxidative stress led to mutational damage that breaks single or double strands, create protein–DNA cross-linkage [18] (Farrugia and Balzan 2012). Landolfo *et al.,* (2008) [19] findings shows that the stress conditions that occur during hypoxia fermentation in a high-sugar medium cause ROS production and stimulate an antioxidant response, also includes superoxide dismutase, trehalose for oxidative damage defence, and protein degradation for the elimination of damaged proteins. The level of oxidative damage to biological components affects cell survival, membrane integrity, and ethanol generation. To scavenge overly produced ROS, cells use both enzymatic and non-enzymatic antioxidant defence mechanism to counteract endogenous oxidative damage. Enzymatic antioxidants are categorized in to two: ROS scavengers and cellular redox balance regulators. Superoxide dismutase, catalase, and peroxidase are most important ROS-scavenging enzymes, while thioredoxin and glutaredoxin are redox regulators. However, non-enzymatic antioxidants are often small compounds that act as ROS scavengers, such as glutathione [20]. Non-enzymatic compounds like glutathione, in contrast to the enzymatic defence mechanism, play a key part in ROS elimination. Glutathione is the most prevalent minor sulfhydryl molecule that acts as an intracellular antioxidant (g-glutamyl-cysteinyl-glycine) [21]. Yap1 and Skn7, transcription factors in oxidative stress response, are predominantly accountable for oxidative stress-induced transcriptional remodelling. The transcription factor SKN7 has a DNA-binding domain. The transcription factor YAP1 is a basic leucin zipper (bZip). YAP1 and SKN7 both transcript many genes associated with oxidative stress- response such as superoxide dismutase - SOD1, SOD2 and glutathione GSH1, GSH2. HYR1 gene catalyses the synthesis of disulphide bonds in YAP1, preventing YAP1 from being exported from the nucleus and allowing it to accumulate in the nucleus [22].

Osmotic stress

The principal stress that *K. marxianus* cell faces is osmotic stress, which is caused by high concentration of sugar in process of fermentation. Under high sugar stress, cell loss water, thereby, turgor pressure loss occurs that results in shrinkage of cell. Hyperosmolarity primarily damage plasma membrane that increase membrane permeability [23]. The common mechanism of yeast cell to maintain osmolarity in both intracellular and extracellular space by increasing solutes that restore turgor pressure. In budding yeast cells signalling under osmotic stress sent by two pathways which are high-osmolarity glycerol (HOG) and mitogen-activated protein kinase (MAPK) signalling pathway.

The HOG pathway maintained by SLN1 and SHO1 branches. SLN1 is a cytosolic histidine kinase domain that forms part of a phospho-relay signalling system that includes the two regulators, YPD1 and SSK1. In osmotic shock SLN1 activity inhibits when change in turgor pressure detected that enhance level SSK1 un-phosphorylation. That allows SSK1 binds to the MAPK kinase which phosphorylate and activate MAPK1 HOG1 [2]. The SHO1 branch breaks further into HKR1 and MSB2 sub-branches, which include two potential osmo-sensors, the mucin-like transmembrane glycoproteins HKR1 and MSB2 each of which regulates the HOG pathway in its own way [24]. HOG pathway also regulates glycerol synthesis in cell that maintains equilibrium with outer environment of cell that expresses genes such as iso-gene GPD, GPD2 (glycerol-3-phosphate dehydrogenase). Under osmotic stress several other compatible osmolytes of cell upregulate gene expression i.e., trehalose, glycogen [25]. Additional prominent functional classes of osmo-inducible genes, according to transcriptome studies for genes which encodes antioxidants (e.g., TTR1, TRX2, and, CTT1) and Heat Shock Proteins, are important in protecting against oxidative stress and protein breakdown such as HSP12, HSP42, HSP104 [26].

Techniques to develop stress tolerant yeast

Genome shuffling

Genome shuffling is a repetitive recombination strategy for strain development that allows randomized recombination in the genotypes of parent strains related to the targeted phenotypes. Genome shuffling shares several characteristics with traditional strain improvement in that both provide genomic variety and strain selection. The fundamental advantage between these two procedures is that the genome shuffling is sexual, resulting in complete populations of better strains, whereas the old method does not. Furthermore, when compared to the traditional strategy, genome shuffling is a faster and more efficient method of creating needed phenotypes. Furthermore, genome shuffling can cause random mutations addressing complicated traits over the whole genome without obtaining genome sequencing data or target strain genomic network [27]. Usually, strain improvement via genome shuffling begins with repetitive mutagenesis of an initial microbial population, as a result, the appropriate mutants are chosen from parental population. In next of genome mutagenesis of strain population their protoplasm preparation needs to do with recursive protoplasm fusion that help in screening and shuffling of developed strain. Strain which develops by genome shuffling have been used for many biotechnological product developments such as antibiotics, bio-ethanol, enzymes (i.e., lipase and protease), vitamins (riboflavin) and many more that are being used in industrial scale [28]. Biot-Pelletier *et al.,* (2018) [29] evolved yeast by genome shuffling and found that exhibit clusters of mutations with high correlations, implying widespread genetic hitchhiking. The presence of pre-existing founder mutations reveals significant driving mutations and harmful hitchhikers based on apparent selection patterns and direct phenotypic assay results.

Global transcription machinery engineering (gTME)

The gTME approach employs a randomly changed form of global transcriptional factor, which regulates the transcription of a huge number of genes and then selects mutants by cultivating them in a stressful environment, gives an interesting example of concurrent manipulation of many genes [30]. Zhao *et al.,* (2014) [31] reported that gTME employed for improving oxidative stress tolerance caused by H_2O_2. The tata binding protein associated factor (TAF25) related mutation was performed, which significantly enhanced its oxidative stress tolerance. However, during fermentation with mutant strain, it was found with shorter lag phase than control strain with enhanced fermentation efficiency. Junhuaa and Suna (2017) [32] enhances ethanol tolerance of *S. cerevisae* by gTME technique, in which suppressor of ty insertion (SPT15) gene was targeted for creating 80 gene mutation and SPT15 gene over expressed in presence of $MnCl_2$. Thereby in presence of 3% of $MnCl_2$, ethanol titer increased by 60.24% compare to control.

Single gene manipulation

For the rational regulation of a specific trait, deletion or upregulation of a particular gene is frequently used. Despite decades of research into the physiological and metabolic grounds of yeast stress tolerance, the genetic foundation of yeast stress tolerance appears to be exceedingly complex, and modifying yeast at the genomic level to enhance its stress tolerance still faces significant obstacles. Multiple studies have shown that single gene alteration can improve yeast stress tolerance. Oh *et al.,* (2019) [33] studied that under acetic acid stress, overexpression of the radiation sensitivity complementing kinase gene (RCK1) significantly improved glucose and xylose sugar metabolism, however glucose fermentation in the presence of acetic acid, the RCK1-overexpressing strain produced twice as much ethanol as the parent strain. Chen *et al.,* (2016) [34] reported that in transgenic yeast, upregulation of whiskey gene (WHI2) increased fermentation capacity of glucose, xylose with acetic acid stress while in glucose fermentation with acetic acid stress, the WHI2-overexpressing strain found with 5-folds greater specific ethanol production than control. In another study, *S. cerevisiae* strain engineered for heterologous lactase dehydrogenase (LDH) gene to produce lactic acid using pre-treated spent coffee grounds (SCG) and hemicellulose as substrate in liquid phase their simultaneous saccharification and fermentation result shows 413% higher yield of lactic acid [35]. Increasing expression of multicopy suppressor (MSN2) gene in *S. cerevisiae.* acquired ethanol tolerance and produce higher ethanol than control strain, MSN2 gene is a transcription factor activated by all stress faced during fermentation including ethanol stress [8].

Adaptive evolutionary engineering

Variation and selection are used in evolutionary engineering to follow nature's engineering principle. As a result, if a targeted phenotype appears accessible to direct or indirect selection, it is an alternative strategy for strain development and process optimization that delivers strong scientific and economic benefits. Adaptive evolution is an essential scientific strategy for studying evolutionary phenomena in a controlled environment [36]. A microorganism is cultured under carefully defined circumstances for longer durations, ranging from weeks to years, to choose superior phenotypes during microbial adaptive evolution. Microbial cells have several economic benefits for adaptive evolution research: (a) Generally microbial cells have basic nutrients needs, (b) these are conveniently maintained in the laboratory, and (c) microbial cells grow rapidly and can be cultured for countless generations in weeks or months with typical cell growth rates. The key reason and benefits of using adaptive evolutionary engineering to increase the features of microorganism is that it does not involve the insertion of a foreign/ recombinant gene into the organism of interest to evolve natural trait of that organism. This method effectively copies the nature by mutating the microorganism's own genes at random, then selecting the desired phenotype under the right conditions. Unlike recombinant DNA technologies, evolutionary solutions in food bioprocess engineering have a better chance of public acceptance because they are more "natural". The major evolutionary engineering applications using yeasts are divided into two categories: (1) substrate utilisation and product synthesis, and (2) stress resistance. Takagi *et al.,* (2005) [37] reported that adapted strain contains 5 time more proline than parent strain without affecting the fermentation profiles and cells that accumulated proline, has higher cell viability than parent strain therefore, it can be conferred that proline may provide tolerance to *S. cerevisiae* under ethanol stress. Kitichantaropas *et al.,* (2016) [38] reported that thermotolerant *S. cerevisiae* strain was found to be multi-stress tolerant (osmotic, oxidative, ethanol, heat) due to its continuous upregulation of trehalose and heat shock protein that helped in developing tolerance to multiple stress. Saini *et al.,* (2017) [39] shows improved ethanol production by 17.5%, by increasing lactose utilization using evolutionary engineering after adapting the strain from 5% to 20% lactose in whey medium, the differential gene expression was 5-folds up-regulated for trehalose genes TPS1, TPS2 and 7-folds up-regulated glycerol gene GPD1, GPD2 after adaptation. Mo *et al.,* 2019 [40] conducted the study on ethanol tolerance using *K. marxianus*, from 6% to 10% (v/v) ethanol tolerant in 100 days and the strain found with enhances multi-stress tolerance compare to parent strain. Pattanakittivorakul *et al.,* (2022) [41] conducted study on adaptation of *K. marxianus* DMKU 3-1042 to enhance the tolerance to high temperatures, which notably improved its ethanol yield by 42%.

CONCLUSION

In the conclusion, the actual ethanol fermentation, yeast strains that are resistant to many stress factors are extremely beneficial for producing ethanol efficiently. The findings of research on the cellular mechanisms of *K. marxianus* required for acquiring tolerance to the various stresses experienced during fermentation have given useful insights for enhancing *K. marxianus* stress tolerance using genetic engineering approaches. It is crucial to emphasise the complicated networks that govern stress-adaptive responses have not been fully understood. When compared to

adjusting to a single stress factor, adaptive evolution shows potential for improving strain performance across several stressors, making it a significant tool for providing multi-stress tolerant yeast strain for industrial application. Nonetheless, more research into stress tolerance pathways is required to enable successful genetic modification of yeast strains capable of withstanding more than single stress.

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Contributions
Upma Pal: writing, reviewing, editing, concept
Sumit pal: editing, reviewing

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Conflict of interest
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