ISSN Print: 2664-844X
ISSN Online: 2664-8458
NAAS Rating (2026): 4.97
IJAFS 2026; 8(8): 94-100
www.agriculturaljournals.com
Received: 10-05-2026
Accepted: 16-06-2026

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Genetic engineering of *Saccharomyces cerevisiae* for enhanced stress tolerance in industrial bioethanol production

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DOI: https://www.doi.org/10.33545/2664844X.2026.v8.i8b.1761

Abstract
Bioethanol is one of the most promising renewable alternatives to fossil fuels, with *Saccharomyces cerevisiae* serving as the principal industrial microorganism because of its high fermentation efficiency, genetic tractability, and long history of industrial application. However, industrial fermentation exposes yeast cells to multiple stresses, including ethanol toxicity, elevated temperature, osmotic stress, oxidative stress, and inhibitory compounds generated during lignocellulosic biomass pretreatment, all of which reduce cell viability and ethanol productivity. Recent advances in molecular biology, synthetic biology, and systems biology have significantly improved our understanding of the genetic and physiological mechanisms governing stress adaptation in *S. cerevisiae*. This review summarizes the major molecular mechanisms underlying stress tolerance, including stress signalling pathways, membrane homeostasis, antioxidant defence systems, and transcriptional regulation. It also discusses recent genetic engineering strategies such as metabolic engineering, CRISPR/Cas9- mediated genome editing, transcription factor engineering, adaptive laboratory evolution, and multi-omics-assisted strain development for constructing robust industrial yeast strains. Furthermore, the review highlights emerging approaches integrating systems biology and genome engineering to improve ethanol yield, fermentation stability, and industrial robustness under multiple environmental stresses. Future prospects focusing on multigene engineering, regulatory network optimization, and systems-level metabolic engineering are also discussed for developing next-generation yeast strains capable of sustaining efficient bioethanol production under industrial conditions.

Keywords: Saccharomyces cerevisiae, bioethanol, stress tolerance, crispr/cas9, metabolic engineering, industrial fermentation

1. Introduction
The increasing global demand for sustainable energy and the urgent need to reduce greenhouse gas emissions have accelerated the transition from fossil-based fuels to renewable biofuels. Among the various renewable energy alternatives, bioethanol has emerged as one of the most widely produced liquid biofuels due to its compatibility with existing transportation infrastructure, high octane value, and potential to reduce carbon emissions. Advances in industrial biotechnology have further promoted the utilization of lignocellulosic biomass as a sustainable feedstock for second-generation bioethanol production, reducing dependence on food-based substrates while supporting circular bioeconomy initiatives. Despite these advances, the economic feasibility of large-scale bioethanol production remains constrained by microbial performance under industrial fermentation conditions, necessitating the development of robust microbial cell factories capable of maintaining high productivity under multiple environmental stresses (Benjamin *et al.*, 2020; Fernandes *et al.*, 2022; Nakamura and Shima, 2018) [1, 2, 3].
Among industrial microorganisms, *Saccharomyces cerevisiae* has remained the preferred ethanologenic yeast owing to its rapid fermentation kinetics, high ethanol productivity, wellcharacterized genetics, Generally Recognized as Safe (GRAS) status, and long history of industrial application. The yeast efficiently converts fermentable sugars into ethanol while tolerating acidic environments and relatively high ethanol concentrations, making it indispensable for large-scale bioethanol production. Furthermore, the availability of extensive genomic resources, advanced molecular biology tools, and efficient genome-editing technologies has established *S. cerevisiae* as an ideal model organism for metabolic engineering and synthetic biology research. However, despite its inherent robustness, industrial fermentation exposes yeast cells to complex environmental challenges that substantially reduce fermentation efficiency and

overall ethanol yield (Liu *et al.*, 2021; Li *et al.*, 2022) [4, 5].

During industrial fermentation, *S. cerevisiae* encounters a combination of abiotic stresses, including high ethanol concentrations, elevated temperatures, osmotic pressure generated by concentrated sugar substrates, oxidative stress, nutrient limitation, and toxic compounds released during lignocellulosic biomass pretreatment such as furfural, hydroxymethylfurfural (HMF), organic acids, and phenolic derivatives. These stresses rarely occur individually but instead act simultaneously, disrupting membrane integrity, protein folding, mitochondrial function, intracellular redox homeostasis, and central carbon metabolism. Consequently, cell viability declines, fermentation rates decrease, and ethanol productivity is significantly compromised. The multifactorial nature of industrial stress therefore represents one of the greatest challenges in developing economically competitive bioethanol production systems (Takagi, 2021; Li *et al.*, 2022; Brandt *et al.*, 2021) [6, 5, 7].

Conventional strain improvement approaches, including random mutagenesis, hybridization, and adaptive laboratory evolution (ALE), have historically contributed to the development of industrial yeast strains with improved stress tolerance. However, these approaches often require extensive screening, generate unpredictable genetic modifications, and provide limited control over complex cellular regulatory networks. The rapid advancement of molecular genetics and synthetic biology has transformed yeast strain engineering by enabling precise manipulation of genes governing stress perception, signal transduction, membrane remodeling, antioxidant defence, transporter activity, and metabolic regulation. Technologies such as CRISPR/Casmediated genome editing, multiplex gene engineering, transcription factor engineering, and systems metabolic engineering have significantly accelerated the development of yeast strains exhibiting enhanced tolerance to multiple industrial stresses while maintaining desirable fermentation characteristics (Gan *et al.*, 2018; Wawro, 2019; Liang *et al.*, 2024) [8, 9].

Recent multi-omics investigations integrating genomics, transcriptomics, proteomics, metabolomics, and genome-wide association studies have considerably expanded our understanding of the molecular basis of stress adaptation in *S. cerevisiae*. These studies have identified numerous genes and regulatory pathways involved in membrane homeostasis, trehalose metabolism, reactive oxygen species detoxification, ion transport, and transcriptional regulation that collectively determine cellular resilience under industrial fermentation conditions. Importantly, the integration of multi-omics datasets with precision genome-editing technologies has enabled the rational design of yeast strains capable of simultaneously tolerating multiple environmental stresses, thereby overcoming limitations associated with single-gene engineering strategies (Ho *et al.*, 2021; Xu *et al.*, 2022; Costa *et al.*, 2023; Tristão *et al.*, 2024) [10, 11, 12, 13].

Although substantial progress has been achieved in engineering stress-tolerant yeast, several challenges remain before genetically engineered strains can be routinely adopted in commercial bioethanol production. Enhanced tolerance to one stress may adversely affect growth, substrate utilization, or ethanol productivity, while laboratory-developed strains frequently fail to maintain equivalent performance under dynamic industrial fermentation conditions. Consequently, future strain improvement efforts require integrated strategies that combine genome editing, adaptive laboratory evolution, systems biology, and computational approaches to optimize multiple stress-response pathways simultaneously (Jacobus *et al.*, 2024; Yuan *et al.*, 2024; Özel *et al.*, 2024) [14, 15, 16].

This review summarizes recent advances in understanding the molecular mechanisms underlying stress tolerance in *Saccharomyces cerevisiae* and critically discusses contemporary genetic engineering strategies employed to enhance yeast robustness for industrial bioethanol production. Particular emphasis is placed on metabolic engineering, transcription factor engineering, CRISPR/Cas-based genome editing, adaptive laboratory evolution, and multiomics-guided strain improvement. In addition, current challenges and emerging research directions are highlighted to provide insights into the development of next-generation industrial yeast strains for sustainable bioethanol production.

2. Industrial Stresses Affecting Saccharomyces cerevisiae During Bioethanol Production

The commercial production of bioethanol involves large-scale fermentation processes that expose *Saccharomyces cerevisiae* to a wide range of environmental and physiological stresses. Unlike laboratory fermentations, industrial processes operate under high substrate concentrations, prolonged fermentation periods, elevated temperatures, and increasing ethanol accumulation, often in the presence of inhibitory compounds generated during lignocellulosic biomass pretreatment. These adverse conditions collectively impair cellular metabolism, reduce cell viability, and ultimately decrease ethanol productivity. Since several stress factors occur simultaneously, industrial yeast strains require broad-spectrum tolerance rather than resistance to a single environmental challenge (Li *et al.*, 2022; Fernandes *et al.*, 2022) [5, 2].

2.1 Ethanol Stress

Ethanol toxicity is one of the primary factors limiting fermentation efficiency. As ethanol accumulates during fermentation, it disrupts the structural organization of the plasma membrane, increasing membrane fluidity and permeability. These changes impair nutrient transport, enzyme activity, and mitochondrial function while disturbing intracellular pH homeostasis. High ethanol concentrations also promote protein denaturation and excessive production of reactive oxygen species (ROS), leading to oxidative damage and premature cell death. Consequently, prolonged exposure to ethanol reduces fermentation rates and lowers final ethanol yield. Yeast cells respond by modifying membrane lipid composition, accumulating compatible solutes such as trehalose, and inducing stress-responsive proteins that preserve cellular integrity under ethanol stress (Takagi, 2021; Li *et al.*, 2022; Singh *et al.*, 2023) [6, 5, 17].

2.2 Thermal Stress

Industrial fermentations frequently operate at elevated temperatures, particularly during simultaneous saccharification and fermentation processes where optimal enzyme activity requires higher operating temperatures. Excessive heat destabilizes membrane lipids, denatures proteins, impairs enzyme activity, and increases intracellular ROS accumulation. Elevated temperatures also reduce cell viability and shorten fermentation lifespan, necessitating costly cooling systems in industrial plants. Thermotolerant yeast strains therefore offer significant economic advantages by reducing cooling costs, minimizing microbial contamination, and improving ethanol productivity under high-temperature fermentation conditions.

Recent studies have shown that alterations in membrane composition, ergosterol biosynthesis, and trehalose metabolism play important roles in enhancing thermotolerance (Yang *et al.*, 2023; Caspeta *et al.*, 2019; Zhang *et al.*, 2023) [18, 19, 20].

2.3 Osmotic and Oxidative Stress

During the initial stages of fermentation, high sugar concentrations create hyperosmotic conditions that result in cellular dehydration, ionic imbalance, and reduced metabolic activity. To counter osmotic stress, *S. cerevisiae* activates the High Osmolarity Glycerol (HOG) signalling pathway, leading to intracellular glycerol accumulation and restoration of osmotic balance. Simultaneously, ethanol accumulation and heat stress stimulate the excessive generation of ROS, which damage proteins, nucleic acids, lipids, and mitochondria. Activation of antioxidant defence systems, including glutathione metabolism, catalase, and superoxide dismutase, is therefore essential for maintaining redox homeostasis and sustaining fermentation efficiency under industrial conditions (Jagtap *et al.*, 2018; Takagi, 2021; Costa *et al.*, 2023) [6, 12]

2.4 Inhibitory Compounds from Lignocellulosic Biomass

Second-generation bioethanol production relies on lignocellulosic biomass, which requires physicochemical pretreatment to release fermentable sugars. However, pretreatment generates several toxic compounds, including furfural, 5-hydroxymethylfurfural (HMF), acetic acid, formic acid, and phenolic derivatives. These inhibitors interfere with glycolysis, damage cellular membranes, disrupt intracellular redox balance, and inhibit essential metabolic enzymes, thereby reducing yeast growth and ethanol productivity. Because inhibitor toxicity frequently occurs together with ethanol and thermal stress, engineering tolerance to a single stress factor is insufficient for industrial applications. Instead, modern strain improvement programmes focus on enhancing tolerance to multiple stresses simultaneously (Brandt *et al.*, 2021; Moreno *et al.*, 2022; Li *et al.*, 2022) [7, 21, 5].

Overall, the simultaneous occurrence of ethanol, thermal, osmotic, oxidative, and inhibitor stresses presents a major bottleneck in industrial bioethanol production. These stresses activate interconnected signalling pathways and metabolic networks, making stress tolerance a complex polygenic trait rather than the result of a single genetic determinant. Consequently, understanding the molecular basis of stress adaptation provides the foundation for developing genetically engineered yeast strains with enhanced robustness and fermentation performance.

3. Molecular Mechanisms Underlying Stress Tolerance in Saccharomyces cerevisiae

The ability of *Saccharomyces cerevisiae* to tolerate diverse environmental stresses is governed by a complex regulatory network involving stress perception, signal transduction, transcriptional regulation, metabolic reprogramming, membrane remodeling, and antioxidant defence. During industrial bioethanol production, yeast cells are simultaneously exposed to ethanol toxicity, osmotic stress, elevated temperatures, oxidative stress, and lignocellulosic inhibitors, requiring coordinated physiological responses to maintain cellular homeostasis and fermentation efficiency. Recent advances in genomics, transcriptomics, proteomics, and metabolomics have substantially improved our understanding of these adaptive mechanisms and enabled the identification of genetic determinants associated with enhanced industrial stress tolerance, providing valuable targets for strain engineering (Liu *et al.*, 2021; Ho *et al.*, 2021; Xu *et al.*, 2022) [4, 10, 11, 5].

3.1 Stress Signalling Pathways

Stress adaptation in *S. cerevisiae* is mediated through highly conserved signalling pathways that detect environmental perturbations and regulate the expression of stress-responsive genes. These signalling cascades coordinate osmotic adjustment, membrane stabilization, ion homeostasis, antioxidant defence, and metabolic reprogramming, thereby enabling rapid cellular adaptation under industrial fermentation conditions.

3.1.1 High Osmolarity Glycerol (HOG) Pathway

The High Osmolarity Glycerol (HOG) pathway represents the principal signalling system responsible for osmotic stress adaptation in yeast. Hyperosmotic conditions activate the mitogen-activated protein kinase *Hog1*, which induces the expression of genes involved in glycerol biosynthesis, osmoprotectant accumulation, and membrane stabilization. Intracellular glycerol functions as a compatible solute that restores osmotic balance without disrupting cellular metabolism. In addition to osmotic adaptation, the HOG pathway contributes to tolerance against ethanol and oxidative stress through interactions with other stress-responsive signalling networks, making it an attractive target for engineering industrial yeast strains with improved fermentation performance (Jagtap *et al.*, 2019; Liu *et al.*, 2022; Chen *et al.*, 2024) [22, 5, 23].

3.1.2 cAMP–PKA and Calcineurin Signalling

The cyclic AMP–protein kinase A (cAMP–PKA) pathway regulates nutrient sensing, cellular growth, and stress adaptation by balancing energy metabolism with survival responses. Under stressful conditions, reduced PKA activity promotes activation of stress-responsive transcription factors, allowing cells to redirect metabolic resources toward stress tolerance rather than rapid proliferation. The calcium-dependent calcineurin signalling pathway similarly contributes to cellular adaptation by regulating ion homeostasis, membrane integrity, and cell wall maintenance. Activation of calcineurin stimulates the transcription factor *CRZ1*, which controls genes involved in calcium transport and stress adaptation. Recent transcriptomic analyses have further identified *CRZ1* as one of the regulatory genes associated with enhanced multiple stress tolerance in industrial *S. cerevisiae*, highlighting its importance as a target for strain improvement (Takagi, 2021; Costa *et al.*, 2023; Wang *et al.*, 2022) [6, 12, 24].

3.2 Membrane Homeostasis and Cellular Protection

Maintenance of plasma membrane integrity is essential for sustaining nutrient transport, enzyme activity, and intracellular homeostasis during industrial fermentation. Ethanol and elevated temperatures directly disrupt membrane structure, making membrane remodeling one of the primary adaptive mechanisms contributing to stress tolerance.

3.2.1 Ergosterol Biosynthesis and Membrane Remodeling

Ergosterol is the principal sterol component of the yeast plasma membrane and plays a critical role in maintaining membrane rigidity, permeability, and fluidity. Ethanol-induced membrane disruption stimulates alterations in lipid composition that preserve membrane stability and cellular function. Several ergosterol biosynthetic genes, including *ERG3*, *ERG4*, and

ERG5, have been associated with improved ethanol and thermal tolerance. Likewise, phospholipid biosynthesis regulators such as *INO2* contribute to membrane remodeling and improved resistance to multiple industrial stresses. Engineering membrane lipid metabolism therefore represents an effective strategy for enhancing fermentation efficiency under stressful industrial conditions (Yang *et al.*, 2023; Albillos-Arenal *et al.*, 2024) [18].

3.2.2 Trehalose and Heat Shock Proteins

Trehalose functions as a compatible solute that protects proteins and cellular membranes from denaturation during ethanol, heat, and osmotic stress. Increased intracellular trehalose concentrations stabilize protein structure, preserve membrane integrity, and facilitate rapid recovery following stress exposure. Heat shock proteins (HSPs), particularly members of the

Hsp70 and Hsp104 families, act as molecular chaperones that prevent protein aggregation and promote refolding of damaged proteins. Coordinated accumulation of trehalose and heat shock proteins therefore constitutes a major protective mechanism contributing to thermotolerance and ethanol resistance in industrial yeast strains (Zhang *et al.*, 2023; Jacobus *et al.*, 2024) [20, 14].

3.3 Oxidative Stress Defence

Industrial fermentation generates excessive reactive oxygen species (ROS) through mitochondrial dysfunction, ethanol toxicity, and elevated temperatures. Excess ROS damages proteins, lipids, nucleic acids, and cellular organelles, resulting in reduced viability and impaired fermentation performance. To maintain redox homeostasis, *S. cerevisiae* activates antioxidant defence systems involving glutathione metabolism, catalase, superoxide dismutase, thioredoxin, and peroxiredoxin enzymes. Enhancement of these antioxidant pathways has been shown to improve ethanol tolerance and overall stress resistance, making them attractive targets for engineering robust industrial yeast strains (Singh *et al.*, 2023; Costa *et al.*, 2023; Yuan *et al.*, 2024) [17, 12, 15].

3.4 Transcriptional Regulation of Stress Responses

Stress adaptation is coordinated by transcription factors that regulate large groups of stressresponsive genes and integrate multiple signalling pathways controlling metabolism, membrane homeostasis, protein quality control, and antioxidant defence.

3.4.1 General Stress Regulators

The transcription factors *MSN2* and *MSN4* function as master regulators of the environmental stress response by activating genes associated with osmotic adaptation, carbohydrate metabolism, oxidative defence, and heat tolerance. Likewise, *HSF1* regulates the expression of heat shock proteins during thermal stress, whereas *YAP1* controls antioxidant defence through activation of ROS-detoxifying enzymes. Coordinated regulation by these transcription factors enhances cellular resilience against multiple industrial stresses and has therefore become an important target for metabolic engineering (Takagi, 2021; Costa *et al.*, 2023) [6, 12].

3.4.2 Calcium and Ion Homeostasis Regulators

The transcription factor *CRZ1* plays a central role in calcium signalling by regulating genes associated with ion transport, membrane stability, and cell wall integrity. Recent comparative transcriptomic studies have identified *CRZ1*, together with *ENA5*, as important regulatory determinants contributing to multiple stress tolerance in industrial *S. cerevisiae*. Additional global regulators, including *SPT15*, *IRA2*, and *INO2*, have also been identified through genome-wide analyses and adaptive evolution studies as key contributors to industria robustness. Manipulation of these regulatory genes frequently produces broader physiological improvements than engineering individual metabolic enzymes because they simultaneously influence multiple downstream stress-response pathways (Ho *et al.*, 2021; Xu *et al.*, 2022; Wang *et al.*, 2022; Tristão *et al.*, 2024; Özel *et al.*, 2024) [10, 11, 24, 13, 16].

Collectively, these molecular mechanisms demonstrate that stress tolerance in *S. cerevisiae* is governed by highly interconnected regulatory networks rather than isolated genes. Integration of stress signalling, membrane remodeling, antioxidant defence, and transcriptional regulation enables yeast cells to maintain cellular homeostasis and sustain ethanol production under multiple industrial stresses. These advances provide the molecular foundation for rational genetic engineering strategies aimed at developing robust industrial yeast strains with enhanced fermentation performance.

4. Genetic Engineering Strategies for Improving Stress Tolerance in Saccharomyces cerevisiae

Recent advances in molecular biology and synthetic biology have transformed the development of industrial *Saccharomyces cerevisiae* strains with enhanced stress tolerance. Unlike conventional strain improvement methods that rely on random mutagenesis or extensive screening, modern genetic engineering enables precise manipulation of genes involved in stress sensing, membrane integrity, energy metabolism, redox homeostasis, and transcriptional regulation. These approaches have facilitated the development of yeast strains capable of tolerating multiple industrial stresses while maintaining high ethanol productivity. The integration of genome editing, systems biology, and adaptive evolution has further accelerated the construction of robust industrial yeast cell factories (Gan *et al.*, 2018; Liu *et al.*, 2021; Liang *et al.*, 2024) [8, 4, 9].

4.1 Metabolic Engineering for Enhanced Stress Tolerance

Metabolic engineering has been widely employed to redirect cellular metabolism towards improved stress adaptation while minimizing adverse effects on growth and ethanol production. Early engineering strategies primarily focused on modifying individual genes associated with membrane stability, osmotic regulation, trehalose biosynthesis, and antioxidant defence. Overexpression of genes involved in glycerol and trehalose metabolism improves osmoprotection and protein stabilization under ethanol and thermal stress, whereas engineering glutathione biosynthesis enhances resistance against oxidative damage. Similarly, manipulation of lipid biosynthesis pathways has been shown to preserve membrane integrity under high ethanol concentrations, thereby improving cell survival and fermentation efficiency. Although single-gene modifications frequently improve tolerance to specific stresses, their effectiveness under industrial conditions remains limited because stress responses are controlled by interconnected regulatory networks rather than isolated metabolic pathways (Takagi, 2021; Li *et al.*, 2022; Costa *et al.*, 2023) [6, 5, 12].

4.1.1 Engineering Membrane Lipid Metabolism

The plasma membrane is the primary target of ethanol-induced cellular damage. Consequently, modification of membrane lipid

composition has become an effective strategy for improving ethanol tolerance. Genetic manipulation of ergosterol biosynthetic genes such as *ERG3*, *ERG4*, and *ERG5* enhances membrane rigidity, maintains membrane permeability, and reduces ethanol-induced leakage of intracellular components. Likewise, engineering phospholipid regulatory genes, including *INO2*, promotes membrane remodeling and improves tolerance to elevated temperatures and ethanol concentrations. These modifications enhance nutrient transport and cellular homeostasis, thereby sustaining fermentation performance under stressful industrial environments (Yang *et al.*, 2023; Albillos-Arenal *et al.*, 2024) [18].

4.1.2 Engineering Antioxidant and Osmoprotective Pathways

Oxidative damage is a major consequence of industrial fermentation stress. Overexpression of genes associated with glutathione biosynthesis, catalase, superoxide dismutase, and thioredoxin systems significantly reduces intracellular ROS accumulation and improves ethanol tolerance. Likewise, increasing trehalose and glycerol biosynthesis enhances osmotic balance and protects cellular proteins against thermal and ethanol stress. These modifications collectively improve fermentation stability by preserving enzyme activity, maintaining redox homeostasis, and reducing cellular damage during prolonged fermentations (Singh *et al.*, 2023; Costa *et al.*, 2023; Yuan *et al.*, 2024) [17, 12, 15].

4.2 Transcription Factor Engineering

Because stress tolerance is regulated by coordinated expression of numerous genes, engineering transcription factors has emerged as an efficient alternative to modifying individual metabolic enzymes. Altering a single global regulator can simultaneously influence multiple downstream pathways associated with membrane stability, oxidative defence, carbohydrate metabolism, and ion homeostasis.

4.2.1 Engineering Global Stress Regulators

The transcription factors *MSN2* and *MSN4* regulate the Environmental Stress Response (ESR) by activating genes involved in heat tolerance, osmotic adaptation, carbohydrate metabolism, and antioxidant defence. Likewise, *HSF1* controls heat shock protein expression during thermal stress, whereas *YAP1* regulates genes involved in oxidative stress resistance. Manipulation of these transcription factors has consistently resulted in enhanced tolerance to multiple environmental stresses while maintaining fermentation efficiency. Their pleiotropic regulatory functions make them attractive targets for constructing industrial yeast strains with broad-spectrum stress resistance (Takagi, 2021; Wang *et al.*, 2022) [6, 24].

4.2.2 Engineering Regulatory Genes

Recent genome-wide studies have identified several regulatory genes that contribute to industrial stress tolerance. For example, modification of *SPT15*, a TATA-binding protein, improves ethanol tolerance by globally regulating stress-responsive gene expression. Similarly, engineering *CRZ1*, *IRA2*, *ENA5*, and *INO2* enhances calcium signalling, membrane integrity, ion transport, and lipid metabolism, thereby increasing tolerance to ethanol, osmotic stress, and elevated temperatures. Compared with conventional metabolic engineering, transcription factor engineering frequently provides broader physiological improvements because it simultaneously regulates multiple adaptive pathways (Ho *et al.*, 2021; Xu *et al.*, 2022; Tristão *et al.*, 2024) [10, 11, 13].

4.3 CRISPR/Cas-Based Genome Engineering

The introduction of CRISPR/Cas technology has revolutionized yeast strain improvement by enabling rapid, precise, and multiplex genome editing. Unlike traditional homologous recombination approaches, CRISPR/Cas9 facilitates targeted gene disruption, insertion, replacement, and regulation with high efficiency. The technology has been extensively applied to modify genes associated with ethanol tolerance, heat resistance, inhibitor detoxification, membrane biosynthesis, and carbon metabolism. Multiplex CRISPR systems further allow simultaneous editing of multiple loci, making it possible to engineer complex traits controlled by several genes. More recently, base editing and CRISPR-mediated transcriptional regulation have expanded the toolbox available for fine-tuning gene expression without introducing double-stranded DNA breaks, thereby improving editing precision and minimizing unintended mutations (Gan *et al.*, 2018; Liu *et al.*, 2021; Liang *et al.*, 2024; Khan *et al.*, 2024) [8, 4, 9, 25].

4.4 Adaptive Laboratory Evolution

Adaptive laboratory evolution (ALE) complements rational genetic engineering by selecting yeast populations under continuous exposure to industrial stresses such as high ethanol concentrations, elevated temperatures, osmotic pressure, or lignocellulosic inhibitors. Beneficial mutations accumulated during long-term evolution frequently improve stress tolerance without prior knowledge of the underlying genetic mechanisms. Whole-genome sequencing of evolved strains has identified numerous mutations associated with membrane composition, transcriptional regulation, mitochondrial function, and stress signalling. Combining ALE with CRISPR/Cas genome editing enables rapid validation of beneficial mutations and accelerates the development of industrial strains with improved robustness and fermentation efficiency (Wawro, 2019; Jacobus *et al.*, 2024) [14].

4.5 Systems Metabolic Engineering and Multi-Omics Approaches

The increasing availability of genomics, transcriptomics, proteomics, metabolomics, and genome-wide association studies has transformed yeast strain engineering from single-gene modification to systems-level optimization. Multi-omics analyses provide comprehensive insights into stress-responsive regulatory networks and facilitate the identification of key genetic determinants controlling ethanol tolerance, membrane stability, and oxidative defence. Integration of these datasets with computational modelling and synthetic biology enables rational design of industrial yeast strains exhibiting coordinated improvements across multiple physiological pathways. Systems metabolic engineering therefore represents a promising strategy for overcoming the limitations associated with conventional metabolic engineering and for developing next-generation yeast cell factories suitable for industrial bioethanol production (Ho *et al.*, 2021; Xu *et al.*, 2022; Özel *et al.*, 2024; Yuan *et al.*, 2024) [10, 11, 15, 16].

Overall, genetic engineering has evolved from modifying individual stress-responsive genes to implementing integrated strategies that combine metabolic engineering, transcription factor manipulation, CRISPR/Cas genome editing, adaptive laboratory evolution, and systems biology. These complementary approaches have substantially improved the

robustness of *S. cerevisiae* under industrial fermentation conditions and continue to expand the potential of yeast as an efficient platform for sustainable bioethanol production. Nevertheless, translating laboratory-engineered strains into commercial biorefineries remains challenging because of complex stress interactions and scale-dependent physiological responses. These emerging challenges and future opportunities are discussed in the following section.

Table 1: Major genetic engineering strategies employed to improve stress tolerance in Saccharomyces cerevisiae for industrial bioethanol production.

Strategy	Target gene(s)/Pathway	Stress addressed	Major outcome	Representative references
Metabolic engineering	ERG3, ERG4, ERG5	Ethanol, thermal	Improved membrane integrity and ethanol tolerance	Yang et al., 2023[18]; Albillos-Arenal et al., 2024
Metabolic engineering	Trehalose and glycerol biosynthesis pathways	Ethanol, osmotic, heat	Enhanced osmoprotection, protein stabilization and cell survival	Zhang et al., 2023[20]; Takagi, 2021
Antioxidant engineering	Glutathione metabolism, catalase, superoxide dismutase	Oxidative, ethanol	Reduced ROS accumulation and improved redox balance	Singh et al., 2023[17]; Costa et al., 2023[12]
Transcription factor engineering	MSN2, MSN4	Multiple stresses	Activation of global environmental stress response	Wang et al., 2022[24]
Transcription factor engineering	HSF1	Heat stress	Increased heat shock protein expression and thermotolerance	Takagi, 2021
Transcription factor engineering	YAP1	Oxidative stress	Enhanced antioxidant defence and oxidative stress resistance	Wang et al., 2022[24]
Regulatory gene engineering	CRZ1	Calcium, ethanol	Improved ion homeostasis and cell wall integrity	Ho et al., 2021[10]
Regulatory gene engineering	SPT15	Ethanol, multiple stresses	Global regulation of stress-responsive genes and enhanced ethanol tolerance	Xu et al., 2022[11]
Membrane engineering	INO2	Ethanol, heat	Improved phospholipid metabolism and membrane remodeling	Albillos-Arenal et al., 2024
CRISPR/Cas9 genome editing	Multiple target genes	Multiple stresses	Precise genome editing and rapid strain improvement	Gan et al., 2018[8]; Liang et al., 2024[9]
Multiplex genome editing	Multiple loci	Complex stress traits	Simultaneous modification of several stress-responsive genes	Liang et al., 2024[9]
Adaptive laboratory evolution (ALE)	Genome-wide adaptive mutations	Ethanol, heat, inhibitors	Selection of industrially robust strains	Wawro, 2019; Jacobus et al., 2024[14]
Systems metabolic engineering	Multi-omics-guided pathways	Multiple stresses	Integrated optimization of cellular metabolism and stress tolerance	Ho et al., 2021[10]; Özel et al., 2024

5. Conclusion

Saccharomyces cerevisiae continues to be the preferred microbial platform for industrial bioethanol production due to its robust fermentation capacity and genetic amenability. However, multiple environmental stresses remain major constraints to efficient ethanol production. Advances in genetic engineering, including metabolic engineering, transcription factor manipulation, genome editing, adaptive laboratory evolution, and systems biology, have significantly improved yeast stress tolerance and fermentation performance. Integrating these approaches with emerging omics technologies and computational tools will facilitate the development of next-generation industrial yeast strains, thereby enhance sustainable bioethanol production and support the transition toward a resilient bio-based economy.

Acknowledgements

The authors also thank the AICRP on EAAI (Bioconversion Technology), University of Agricultural Sciences, Dharwad for providing institutional support and encouragement during the preparation of this review manuscript.

Conflict of interest

Authors have declared that no competing interests exist.

Authors' contributions

Shubha S. conceived and designed the review, performed the literature survey, critically analyzed the published literature, drafted the manuscript, prepared the tables, and supervised the overall work. Naveen V. assisted in literature collection, manuscript preparation, formatting, and critical revision. Arun Y. P. contributed to the scientific review of the manuscript and provided valuable technical inputs. Santosh G. P. assisted in manuscript editing and literature validation. Gagan Kumar M. contributed to manuscript revision and language editing. All authors read and approved the final manuscript.

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