Strategies for Overcoming the Presence of the Acetic Acid Inhibitor in the Production of 2G Ethanol

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ABSTRACT

The presence of the inhibitor acetic acid in lignocellulosic hydrolysate represents one of the major challenges in the production of 2G ethanol. This study seeks to review the most recent research on genetic manipulation of industrial strains of *S. cerevisiae* in order to increase their tolerance to the inhibitor acetic acid. The research focuses on understanding the mechanisms of response to acetic acid stress in cells and the current advancements in metabolic and genetic engineering that contribute to the development of more competitive strains in this industry.

Keywords: acetic acid, tolerance, *Saccharomyces cerevisiae*, cellulosic ethanol, industrial strains

1 INTRODUCTION

Brazil is currently the largest producer of 2G ethanol in the world, possessing the largest 2GE plant capable of producing 82 million liters per year (Bonfim Bioenergy Park - Guariba, SP). 2G ethanol is produced from the by-products of ethanol and sugar production (sugarcane straw and bagasse). The biomass is mainly composed of cellulose, hemicellulose, and lignin. Pretreatment with sulfuric acid[1] or organosolv[2] are considered to be the most efficient way of removing lignin and releasing pentoses such as xylose from hemicellulose. However, these pretreatments also generate by-products such as hydroxymethylfurfural (HMF), furfural and acetic acid, that inhibit the productivity and growth of *S. cerevisiae*, the yeast most commonly used to produce this biofuel.

To overcome the problem of the presence of inhibitors in the hydrolysate, the Melle-Boinot process can be applied, which combines high cell concentrations and a fed-batch process, allowing manipulation of the feed flow rate and control of the hydrolysate concentration[3]. In addition, it is possible the integration of the first and second generation processes (1G2G) to produce ethanol from biomass, in order to minimize problems related to the high concentration of inhibitory compounds in the medium[4]. Thus, a third option to ensure that inhibitors in lignocellulosic hydrolysate are no longer a challenge to the production of 2G ethanol is to develop a fermenting microorganism capable of tolerating these compounds during fermentation.

Acetic acid is the inhibitor usually found in the greatest amount in hemicellulose hydrolysates. Its inhibitory activity is related to the pH of the medium and the dissociation constant of the acid (*pKa*). As the pH of the medium decreases, the concentration of the acid in its undissociated and lipophilic form increases, favoring the diffusion of the acid across the cell membrane of the fermenting microorganism[5]. Once in the cytoplasmic environment, it dissociates into the acidic form, which can lead to inhibition of growth and metabolic activity, as well as other deleterious effects, including oxidative damage to the membrane and energy depletion[6]. The toxicity of acetic acid to *S. cerevisiae* yeast is particularly severe under conditions where the pH of the extracellular medium is lower than the *pKa* of the acid (*pKa* = 4.76)[5], near from the pH of the fermentation process (pH 5.5 - 4.5).

Studies of the *S. cerevisiae* transcriptome have revealed that acetic acid tolerance is controlled by multiple genes whose interactions are highly complex and are responsible for different cell responses (**Figure 1)**. However, most research focuses on the genetic modification, suppression, or overexpression of genes related to this mechanism in laboratory strains. The genetics of industrial *Saccharomyces* strains are more complex than those of laboratory reference strains, as they can be diploid, aneuploid or even polyploid[7]. One of the advantages of developing genetically modified yeast strains for industrial applications, therefore, is the use of strains naturally adapted to different industrial stress conditions, especially the osmotic stress caused by high sugar concentration[7]. This is, in fact, one of the differentials of *S. cerevisiae*, which is widely recognized for its high fermentative performance even at sugar concentrations as high as 200 g/L[8].

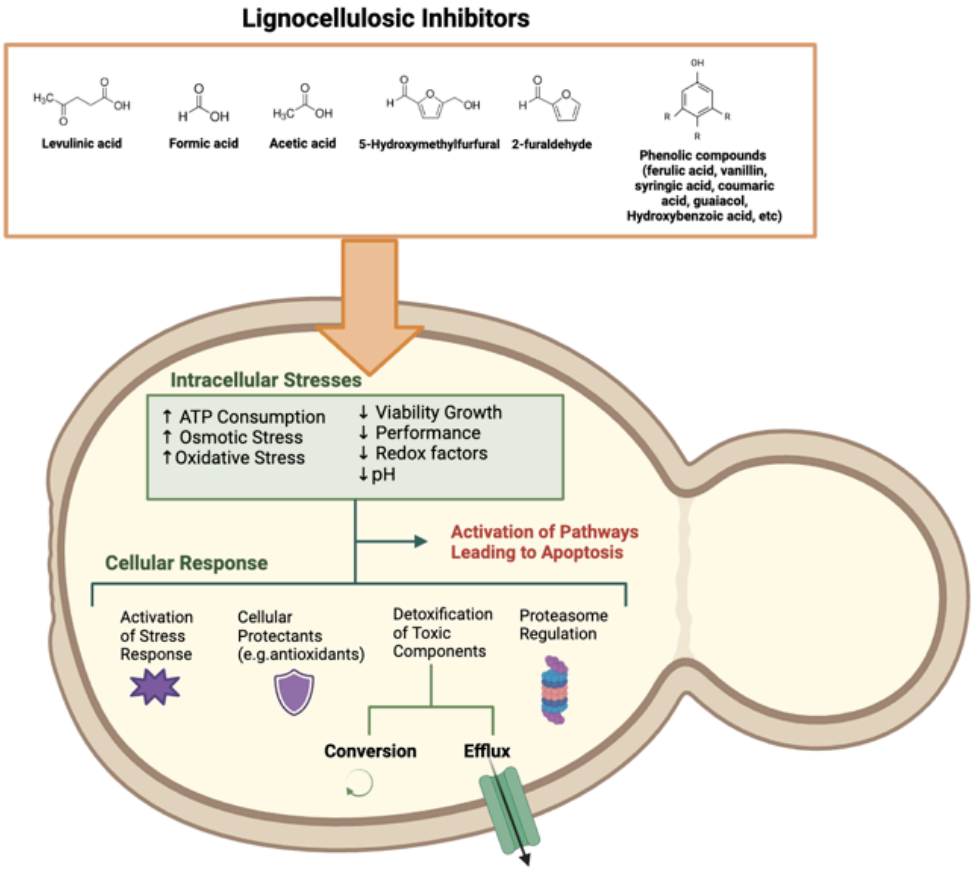

Figure 1 Overview of lignocellulosic inhibitors present in hydrolysates and the cellular stress responses. Adapted from[9]

This work seeks to carry out a bibliographical review of the most recent articles involving the metabolic and genetic engineering of industrial strains of *S. cerevisiae* with the aim of increasing their tolerance to acetic acid. The parameters of pH, the maximum concentration of acetic acid in g/L tolerated by the engineered strains, and the specific methodology used in each case were evaluated.

2 MATERIAL & METHODS

The research was based on a systematic review using the Google Scholar and Saccharomyces Genome Database (SGD) tools, and was carried out using the following keywords: acetic AND acid AND tolerance AND saccharomyces AND cerevisiae AND cellulosic AND ethanol AND industrial, AND strain. The search resulted in 8,400 papers from Google Scholar, 5 from PUBMED and 322 from PUBMED Central. A total of 6 papers from the last five years were selected. Papers that did not address strategies involving industrial strains of *S. cerevisiae* were excluded.

3 RESULTS & DISCUSSION

Table 1 Acetic acid concentrations, medium pH, and industrial yeast strains in the cited studies

Yeast Strain	Acetic acid (g/L)	pH	Reference
S. cerevisiae 4126	5.0	3.5	Lamour et al (2019)[10]
KE6-12	6.06	4.5	Cámara et al (2020)[11]
TF2	6.0	4.5	Brandt et al (2021)[12]
JDY-01	2.5	8.0	Tadioto et al (2022)[8]
PLY01-GPX1	5.0	4.0	Ye et al (2022)[13]
S. cerevisiae 4126	5.0	3.6	Xiong et al (2024)[14]

Table 2 Genes that improve acetic acid tolerance in industrial yeast strains

Gene	Function	Methodology	Strain background	Reference
YHB1	Nitric oxide reductase	Overexpression[a]	*S. cerevisiae* 4126	Lamour et al (2019)[10]
SSK2	Protein kinase	Repression[b]	KE6-12	Cámara et al (2020)[11]
TAL1+FDH1	Transaldolase and Formate Dehydrogenase	Overexpression[a]	CelluXTM1	Brandt et al (2021)[12]
TAL1	Transaldolase	Overexpression[a]	JDY-01	Tadioto et al (2022)[8]
AKL1	Protein kinase	Overexpression[c]	PLY01-GPX1	Ye et al (2022)[13]
CAR1	Arginase	Overexpression[a]	*S. cerevisiae* 4126	Xiong et al (2024)[14]

[a] Homologous Recombination, [b]Transcriptional modulation by CRISPRi, [c] CRISPR-Cas9 genetic editing

As illustrated in **Figure 1**, the active transport-mediated removal or elimination of inhibitory compounds might pose an additional energy demand on cells. Simultaneous intracellular buildup of these substances leads to the generation of reactive oxygen species (ROS)[15]. Yeasts that are subjected to oxidative stress usually generate ROS scavengers and initiate pro-survival mechanisms such as programmed cell death and mitophagy.

All the investigations cited in **Table 1** and **Table 2** have focused on creating strains capable of fermenting lignocellulosic hydrolysates, although the biomass sources and methodology vary. As already mentioned, many genes are linked to different cellular response mechanisms for acetic acid tolerance. Protein kinase genes (SSK2 and AKL1) are important for cell activation processes within signaling pathways because they regulate enzymes that catalyze the phosphorylation of proteins by transferring a phosphate group from ATP[16,17]. Transaldolases (TAL1), on the other hand, are important for the non-oxidative branch of the pentose phosphate pathway and are located in the cytosol, having been implicated in broader phenotypes of resistance to multiple inhibitors[18]. Nitric oxide reductases (YHB1) are also involved in the response to reactive nitrogen species, oxidative stress, and misfolded proteins; they are located in the nucleus, mitochondria, and stress granules[19].

4 CONCLUSION

Numerous investigations have attempted to enhance the fermentative efficiency of microbial cell factories and understand the effects of lignocellulose inhibitors in light of the growing interest in the fermentation of lignocellulosic hydrolysates. It is challenging to direct the improvement of strains given the degree of particular information available. Thus, the main objective of this review was to gather the published experimental findings that describe genetic alterations (gene suppression or overexpression) that impact *S. cerevisiae's* ability to withstand the inhibitor acetic acid present in lignocellulosic hydrolysates.

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