Molecular Mechanisms Involved in Yeast Fitness for Ethanol Production

Elis Cristina Araujo Eleutherio*, Fernanda Cigagna Boechat, Rayne Stfhany Silva Magalhães, Germana Breves Rona and Aline Araújo Brasil

Department of Chemistry, Federal University of Rio de Janeiro, Brazil

Submission: December 01, 2018; Published: February 13, 2019

*Corresponding author: Elis Eleutherio, Department of Chemistry, Federal University of Rio de Janeiro Av Athos da Silveira Ramos, Brazil

Abstract

Saccharomyces cerevisiae is well adapted to alcoholic fermentation. In this yeast, fermentation predominates over respiration at high glucose concentrations even under the presence of oxygen. Besides the solid fundamental knowledge base in system biology and in yeast metabolic pathways, this microorganism offers many favorable bioprocessing traits which allow robustness under industrial conditions, such as:

a. Genetic accessibility
b. High tolerance to fermentative stresses - high temperatures, high ethanol and sugar concentrations, low pH
c. Ideal physiology features (larger cell size, non-pathogenic, short generation time and ability to grow in a highly reproducible and genetically stable way with poor nutrient requirements). In this chapter, we will review the sugar and ethanol metabolism regulation as well as highlight the main molecular mechanism contributors to yeast multiple stress tolerance during fermentation. Furthermore, we will also discuss the future direction of genome and metabolic engineering of yeasts for ethanol production.

Keywords: Ethanol; Glucose; Metabolism; Stress response; Fermentation; Saccharomyces cerevisiae

Abbreviations: PKA: Protein Kinase A; ROS: Reactive Oxygen Species; HSP: Heat Shock Proteins; XR: Xylose Reductase; XDH: Xylitol Dehydrogenase; XI: Xylose Isomerase; ETC: Electron Transport Chain; CAC: Citric Acid Cycle

Introduction

Ethanol is one of the most important fuels in the actuality, especially because through the last years, the worries with the environment are established and growing and the sustainability has been one of the biggest highlights in the industries philosophy. The fossil fuels have been used by humanity for centuries, but it is changing while the concern with the use of these fuels is growing. That is because fuels, such as oil and coal, are non-renewable and finite, what makes the exploration increasingly harder and more expensive, and is extremely polluting and aggressive to environment. Therefore, the idea of the use of renewable resources that are considered greener, being less aggressive to the environment, is growing. One of the biggest spotlights in this manner, is the production of ethanol which can be performed from renewable sources, is less polluting and can be produced through raw material that are residues of the agroindustry and are, currently, often used as routed to lower value-added applications.

First-generation ethanol is ethanol produced from sugar and starch sources, such as sugarcane and corn. In the case of sugarcane, for example, the plant is processed and the sucrose available in the juice is metabolized to monosaccharaides, glucose and fructose, which are promptly fermented. This process is well established and very viable and the most widely used in terms of ethanol production [1]. However, it has some limitations. One of them is, because it is necessary to plant the raw material for the extraction of sugar and then the fermentation and production of ethanol from it, large tracts of arable soil that could be producing food are used in the production of energy. This is a problem that has been raised for the future, as the world’s population is growing a lot and all food production capacity will be needed if the population continues to grow faster than the availability of food. In addition, in the processing of sugarcane for the withdrawal of the juice to produce ethanol, an enormous amount of waste (the sugarcane bagasse), made of cellulose, hemicellulose and lignin, is generated, which is not good for the environment. However, this waste can be harnessed in a more interesting way from the energetic and environmental point of view, by using it to produce more ethanol.

Ethanol generated through bagasse and other waste is called second-generation ethanol. This biomass is a much more complex raw material that needs some other treatments before fermentation. A pretreatment is necessary for making carbohydrates more readily available for hydrolysis, which is responsible for
transforming long chains of carbohydrates into fermentable sugars. The pretreatment may be biological, chemical, mechanical or physical-chemical, while the hydrolysis may be enzymatic or acidic [2]. This is a very interesting process because it adds a value to a waste that would have been destined to a less noble process, such as direct burning for energy, and it is an environmentally friendly strategy. However, like every process, it also has limitations. Currently the pretreatment is usually carried out through physical-chemical treatment (use of acids and temperature, by example) that requires a post-neutralization treatment of the medium so that the subsequent enzymatic treatment for hydrolysis and the fermentation itself does not find a hostile environment and ethanol production can occur.

This process is quite expensive, because there is a high expense with this neutralization besides all the controls that the process implies, like the temperature control. In this respect, an interesting treatment would be biological. However, this treatment is also very expensive due to the conditions that a microorganism demands for it to work optimally, such as pH and temperature control mainly. Moreover, the availability of sugar in the medium is not simple, since these polymers, especially hemicellulose and mainly lignin, are a complex mixture composed of residues of different monosaccharides, linked by different glycosidic bonds, which demands action of different microorganisms to treat this structure that is so difficult to break. Many studies have been conducted to reduce these costs in order to increase the feasibility of using these techniques [3]. The best scenario is the production of first-generation ethanol coupled with second-generation ethanol.

The metabolic regulation behind S. cerevisiae preference for fermentation

The yeast Saccharomyces cerevisiae is the most common microorganism used for alcoholic fermentation in industrial processes [4]. This yeast is one of the simplest eukaryotes and is really versatile, being able to grow at high sugar concentration [5]. The most abundant sugar in nature is glucose, which is also the preferred carbon source of S. cerevisiae. Glucose enters the cell through at least 6 transporters (Hxt1, Hxt2, Hxt3, Hxt4, Hxt6, Hxt7) which have different affinities for sugar and have their expressions modulated by the different concentrations of glucose in the medium [6]. The repression of HXT genes occurs in the absence of glucose through the recruitment of the repressor complex Ssn6-Tup1, with the help of Mth1 and Std1 proteins. The HXT genes are inducible by glucose through inhibition of Mth1 and Rgt1[6]. Other monosaccharides may also be fermented by S. cerevisiae, such as mannose and fructose and, after a period of adaptation, galactose. Sucrose is hydrolyzed preferentially by invertase, located at the surface of the cell.

Figure 1: Simplified scheme of ethanol and glucose metabolism. In the figure are represented the pathways that are important for ethanol production and the pathways which regulation is important so ethanol production can be maximized, such as glycolysis, pentose phosphate pathway, gluconeogenesis, glycogen synthesis and degradation and trehalose synthesis and degradation. The inset represents the simplified scheme of ETC.

The cell gain of energy through glucose can occur in two different ways on substrate-level phosphorylation (fermentation) and oxidative phosphorylation (respiration). Once in cytosol, glucose is immediately phosphorylated to glucose-6-phosphate (G6P) to trap sugars inside the cell and avoid diffusion out the cell. G6P is a cell metabolite of the five major yeast glucose pathways glycolysis, gluconeogenesis, glycogen synthesis and degradation, trehalose metabolism and pentose phosphate shunt (Figure 1) [5]. The glycolytic pathway is composed of 10 reactions, producing two ATP molecules, two NADH molecules and two molecules of pyruvate.

Glucose flux is dependent on the activity of enzymes which catalyze irreversible reactions; the other reactions are close to equilibrium and, thus, reversible. The first of those enzymes is hexokinase, which is responsible for the phosphorylation of glucose to glucose-6-phosphate. Unlike other enzymes in the pathway, hexokinase is not regulated by energy demand, as it may give rise to other destinations besides energy production but seems to be inhibited by trehalose-6-phosphate (T6P) [7]. The second enzyme that regulates the glycolytic flux, and the most important one, is phosphofructokinase1 (PFK1) that converts fructose-6-phosphate to fructose-1,6-bisphosphate. This enzyme is inhibited in an allosteric way by ATP and activated by AMP and fructose-2,6-bisphosphate. Pyruvate kinase is the third enzyme that regulates the glycolytic flux and is also inhibited by ATP [8].

How to cite this article: Elise C A E, Fernanda C B, Rayne S S M, Germana B R, Aline A B. Molecular Mechanisms Involved in Yeast Fitness for Ethanol Production. Adv Biotech & Micro. 2019; 12(5): 555847. DOI: 10.19080/AIBM.2019.12.555847.
Pyruvate can then be directed to two distinct pathways: one oxygen-dependent and one oxygen-independent. If oxygen is present, pyruvate can be transported to the mitochondrial matrix and then converted to Acetyl-CoA, which can be conveyed to the Citric Acid Cycle (CAC; generation of one GTP molecule, three NADH and one FADH$_2$), and then to the Electron Transport Chain (ETC). As electrons are sequentially transferred from NADH/FADH$_2$ to oxygen, protons are translocated from the matrix to the intermembrane space creating an electrochemical gradient which is used as energy to ATP synthesis. In *S. cerevisiae*, NADH and FADH$_2$ render 1.5 ATP each one because yeast lacks complex I [9] (Figure 1). Intramitochondrial NADH is reoxidized by an internal NADH ubiquinone oxireduct (Ndi1). Cytosolic NADH can be reoxidized by the external NADH dehydrogenase (Nde1/Nde2) or via the glycerol-3-phosphatate dehydrogenase shuttle. Neither Nde1/Nde2 nor Ndi1 are proton pumps [10]. Consequently, in *S. cerevisiae*, the complete oxidation of glucose to CO$_2$ yields 22 ATPs. In the absence of oxygen pyruvate can be directed to the synthesis of ethanol. This synthesis occurs in two reactions conversion of pyruvate to acetaldehyde with the release of CO$_2$, catalyzed by pyruvate decarboxylase enzyme and then from acetaldehyde to ethanol through alcohol dehydrogenase [5]. *S. cerevisiae* has seven different isoforms of alcohol dehydrogenase (Adh1-7p) that is an enzyme responsible for the regeneration of NAD+ cytosolic, that is very important to glycolytic pathway, restoring the redox balance when converts acetalddehyde to ethanol. The isoforms have different characteristics such as their affinities for the substrates and their expressions [11]. What rules if pyruvate will be completely oxidized to CO$_2$ (respiration) or converted into ethanol (fermentation) is not the presence of oxygen but sugar concentration.

Thus, at high-glucose concentration, even when oxygen is present, yeast ferments glucose to ethanol. This is called glucose repression (or Crabtree Effect) and it is a very regulated process. During glucose repression, citric acid cycle and electrons transport chain enzymes are not expressed and other enzymes, responsible for glycolysis, are overexpressed. A few different pathways, signaled by glucose, are responsible for this regulation. Figure 2 & 3 provided visual overview of Snf3/Rgt2-Rgt1, Mig1/Snf1/Hxk2 and Ras/cAMP/PKA pathways in the glucose repression and derepression mechanisms. Although glucose repression has been extensively studied, this mechanism is not completely understood.

When glucose concentration falls, CAC and ETC enzymes are derepressed, leading to an expressive reduction in the glycolytic flux. The ATP yields obtained from glucose respiration are around 10 times higher from glucose fermentation causing a higher inhi-
The repression complex is also formed by Snf1 complex, which include Snf1, Snf4, and Gnl83; Snf1 complex is linked to the repression complex through the binding of Snf1 to Hsk2 [21] (2f). Genes which codes for enzymes of CAC, ETC, alternative carbon sources consumption and gluconeogenesis are targets of glucose repression complex [20]. Other pathway involved with glucose repression is the Ras/cAMP/PKA pathway. Glucose interacts with Gpr1, activating the G proteins Gpa2 and Ras (2g), which interact with adenylate cyclase (Cyr1) stimulating cAMP synthesis [22,23] (2h). cAMP binds to the regulatory Bcy1 subunits of PKA, causing the dissociation of the regulatory subunits from the catalytic subunits (TPK), activating PKA [23] (2i). PKA has an important role because it is responsible for the phosphorylation, and thus regulation, of a lot of the enzymes involved in glucose signaling, such as Rgt1 that was previously quoted [19]. Other PKA targets are phosphofructokinase 2, fructose-1,6-bisphosphatase, glycogen synthase, glycogen phosphorylase and trehalase, impairing gluconeogenesis, trehalase and glycogen synthesis and favoring glycolysis [24].

Mth1 and Std1 are recruited, causing a conformation change that makes possible the binding of Rgt1 to its recognition sites in DNA generating transcriptional repression [23] (3a). At low-glucose concentration, Snf1 is phosphorylated, and activated by Sak1; in this form, Snf1 phosphorylates Hsk2 and Mig1 (3b), disassembling the repression complex in the nucleus and leading to Mig1/Hsk2 exportation to the citosol [21] (3c).

**Stress response during alcoholic fermentation in S. cerevisiae**

The ability of S. cerevisiae to overcome other microorganisms and dominates the culture medium during alcoholic fermentation is associated to its high fermentative performance and capacity to withstand the adverse conditions of fermentation processes [25]. During fermentation, cells are submitted to different kinds of stress, such as accumulation of ethanol, high osmolarity, heat and oxidative stress. These stresses can occur in concert or sequentially and have different impacts on yeast cells. The ability of a given yeast strain to respond to these stresses determines its robustness and its performance in industrial processes.

**Ethanol stress**

Ethanol is the final and interest product of fermentation process and also the main stress factor for yeast cells [26]. Even at low concentration, ethanol inhibits cell division, decreasing the growth rate due to the inhibition of glucose and amino acids consumptions [27]. The impact is higher at the end of the fermentation. As ethanol concentration increase, reaching high levels, the membrane permeability is altered, which increases the influx of protons (lowing the pH at the cytosol to toxic levels); proteins are denatured, impairing their functions in cell metabolism; the levels of Reactive Oxygen Species (ROS) increase, leading to an oxidative stress; and the water availability decreases, causing dehydration [27,28]. All of them impact on cell viability, growth rate and ethanol yield [29].

The main targets of ethanol are membranes and proteins, influencing their structure and functions [28]. Ethanol interacts with the lipid bilayer of the plasma membrane through the hydrophilic side, disturbing the original membrane structure [27,30]. The fluidity and integrity of the plasma membrane are important for cell protection; therefore, it is common to detect high levels of Reactive Oxygen Species (ROS) increase, leading to an oxidative stress; and the water availability decreases, causing dehydration [27,28]. All of them impact on cell viability, growth rate and ethanol yield [29].

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glycolytic enzymes, such as hexokinase and pyruvate kinase, impacting directly the fermentation rate [27].

As yeast cells are submitted to ethanol stress, some biochemical mechanisms are activated to improve tolerance against the stressful situation. The most common mechanism that can be detected in yeast cells under heat and ethanol stress are trehalose synthesis and expression of Heat Shock Proteins (Hsps) [31]. The disaccharide trehalose is synthesized in a wide range of organisms, among them yeast, mainly as a response to several stress conditions [33]. Some experiments have shown that the levels of trehalose increase during fermentation, indicating that the mechanism is activated to confer tolerance to the cell in such condition [34]. In addition, it was observed that mutant strains unable to hydrolyze trehalose show increased tolerance to ethanol stress, confirming the importance of this disaccharide to confer robustness during fermentation [35]. This sugar mitigates the denaturing effects of ethanol chaotropicity by stabilizing the structure of membranes and proteins [36]. Trehalose avoids denaturation by excluding water from protein or membranes’ hydration layer and ordering around it. Trehalose-water interaction is stronger than water-water. At high concentration, trehalose competes against biomolecule for the available water, restricting the mobility of the water molecules of the hydration layer which stabilizes biomolecule structure during stress.

*S. cerevisiae* cells also synthesize Hsps for protection against ethanol stress, in the same way that is observed in heat shock. Among the 6 different Hsps analyzed and related to ethanol stress, only Hsp104 and Hsp12 have been related to tolerance to this condition [28]. Hsp104 does not avoid protein denaturation, differently from other chaperones, but preserve protein structures, allowing their reactivation after the end of the stress [37]. It was observed that mutant strains that do not express Hsp12 show low tolerance to ethanol stress [38]. It has been shown that this chaperone is localized only at the plasma membrane, suggesting that Hsp12 is involved with membrane protection.

**Oxidative stress**

Although ethanol production by *S. cerevisiae* is an anaerobic process, cells need O₂ to grow in optimal conditions in the first steps of fermentation and increase ethanol yield. Due this, it is common to name the fermentation as a semi anaerobic process, and, thus, passable to be submitted to oxidative stress [27]. Oxidative stress is characterized by the imbalance between the antioxidant response and ROS (O₂⁻, OH and H₂O₂) [39]. When this happens, the ROS levels increase, inducing negative effects into the cells as damage at the membrane, lipids, proteins, DNA; as a consequence, cell growth is inhibited, and apoptosis is activated [40].

During fermentation, ethanol stress and heat shock can induce an increase in ROS levels or inactivation of antioxidant enzymes [41,42]. Even under anaerobic fermentations, some metabolic pathways require the presence of molecular oxygen; ROS can be produced by NAD(P)H-dependent pathways, such as cytochrome P450 systems [43]. Ethanol reduces biomolecules hydration layer, making them more prone to ROS attack. According to Trevisol et al. [35], yeast cells showed increased levels of lipid peroxidation and protein oxidation after fermentation. ROS can also be produced during the pretreatment process required for the use of lignocellulosic residues in second generation ethanol [44].

**Some studies have shown that strains used to produce bioethanol show high levels of ROS, as well as the up-regulation of some antioxidant compounds**

- The enzymes Cu/Zn superoxide dismutase, which dismutates O₂⁻ to H₂O₂ and O₂, and catalase, which converts H₂O₂ to water and O₂
- glutathione [27,39]. When these defenses are not working, the cells lose the ability to grow, then decreasing the fermentation rate, that means, drop of ethanol production.

In industrial processes, yeast cells are recycled and reused in serial fermentation batches, leading to cell aging and increasing petite concentration which reduces ethanol yields [35]. More stable mitochondria are essential for a better protection against ROS. Overexpression of mitochondrial cytochrome C oxidase chaperone gene (COX20) improved oxidative, acid and ethanol stress tolerance and increased ethanol yield [44].

**Heat stress**

Both first- and second-generation ethanol production use high temperatures in the fermentation of *S. cerevisiae* [46]. Fermentation process for bioethanol production at high temperatures presents some financial advantages. High temperatures avoid contamination, reduce cooling costs and allow simultaneous saccharification and fermentation (this is interesting when ethanol is produced from corn) [47,48]. On the other hand, high temperatures directly affect yeast growth rate and lead to protein denaturation [49]. Yeast cultivation under increased temperatures leads to petite mutation, which also impairs fermentation [35,50].

Heat stress enhance the H⁺-ATPase activity, protein responsible for the active transport of protons across the membrane, leading to an increase in the output of protons from the cell and membrane depolarization [50]. Heat shock causes protein denaturation, besides causing a disorder in the plasma membrane, increasing membrane permeability [50].

Response to thermal stress is activated at temperatures above 35°C, inducing heat shock proteins [50]. Hsp proteins play an important role in folding and refolding proteins, as well as de-
grading misfolding and denatured proteins [51]. The major Hsp involved in the defense against heat shock are Hsp70 and Hsp104. Hsp 70 is involved in the response to thermal stress acting in the aggregate’s prevention and proteins refolding [51]. Hsp104 has a unique characteristic, it rescues inactivated proteins from insoluble aggregates, formed by thermal stress, then these proteins are refolded by other chaperones like Hsp70 [51].

During thermal stress there is an enhance in the antioxidant enzymes activity due to increased production of ROS, raising levels of mitochondrial manganese superoxide dismutase (SOD2 gene) [50], cytosolic copper-zinc superoxide Dismutase (SOD1 gene) [52] and cytosolic catalase T (CTT1 gene) [50]. Other way of yeast cell defends itself against thermal stress is by producing trehalose. It was observed that genes involved in the cytoskeleton, such as SAC6, SHE4, SLA2, SPCC72, are required against heat stress [52]. Other genes were demanded for thermal tolerance like genes involved in transcription (such as MED1, MED2, PAF1, SW13, SW16 and SRB2), RNA processing (such as ISY1, LEA1, LSM6) and actin cytoskeleton (such as SAC6, SHE4, SLA2, SPCC72) [52].

### Osmotic stress

The large amount of sugar found in the fermentation medium to enhance fermentation rate leads to osmotic stress [47,49,52]. This stress leads to a contraction of the cell due to cytoplasmatic water waste, leading to loss of turgor pressure [27,51,52]. In order to balance the osmotic pressure across the membrane the yeast cell produces glycerol, a compatible osmolyte [27,47,52], by the HOG- MAPK pathway (high-osmolarity glycerol-mitogen-activated protein kinase) [52]. Vacuolar protein transport genes, such as VPS1, VPS3, VPS16, genes involved in energy metabolism, such as OXA1, PET100, and SAC1, and cell defense genes such as HOG1 and SOD1, are required in osmotic stress [52].

Msn2 and Msn4 are binding STRE (stress response element) factors involved in the stress response against osmotic stress. Msn2 and Msn4 have an indirect protective role in Hog1 against specific phosphatases in the nucleus [51]. Hog pathway induc- es GDP1 and GPP2, under osmotic stress, leading to glycerol production [51]. Gpd1 (glycerol-3-phosphate dehydrogenase) catalyze the conversion of dihydroxyacetone phosphate to glycerol-3-phosphate (G3P), and Gpp2 (glycerol-3-phosphate phosphatases hydrolizes G3P into glycerol [51]. After osmotic shock, the membrane protein Fps1 is closed accumulating glycerol inside the cell [51].

### pH stress

Lignocellulosic hydrolysate of second-generation ethanol fermentation contains furans derivatives and weak acids, which impair fermentation [53]. In first generation ethanol fermentation process, yeast cells must be washed with sulfuric acid in order to avoid contamination by bacteria before be reused in another batch. In that way, yeast can last up to 6 months if no contamination occurs [54]. This process reduces cell viability and ethanol yield [55]. The pH medium affects yeast growth rate, fermentation products [49,53,56], inhibits glycolytic flux and induces oxidative stress [53]. The permeability of some nutrients is dependent on the concentration of H+ in the culture medium [49].

Weak acids have inhibitory effect on cell growth, owing to the fact of non-dissociated acids cross the plasma membrane through passive transport, decreasing cytoplasmatic pH [56,57]. There are two hypotheses to explain the effect of weak acids: intracellular concentration of anions and decoupling [56]. By the decoupling theory, the decrease in intracellular pH leads to a pumping of protons out of the cell by ATPase, plasma membrane, expending ATP [56]. The response to weak acids is dependent on the HOG-MAPK pathway and pathways related to cell wall synthesis [58,59]. The response to inorganic acids is similar to response to weak acids. However, inorganic acid tolerance relies on a mechanism Protein Kinase A (PKA) dependent [58]. In acidic media the yeast activates the general stress response to adapt, already stress tolerance involves the regulation of the cell cycle, decreasing transcription levels and protein synthesis [58]. YGP1, TPS1 and HSP150 are induced in pH stress [60]. It was observed a low increase in trehalose concentration under acid stress in *S. cerevisiae* [53]. Although yeast develops some defense mechanisms, engineering a low pH tolerant cell is one of the targets of second-generation ethanol synthesis [61].

*S. cerevisiae* in sustainable fermentation: development of strains for second generation ethanol

The sustainable bioethanol production from lignocellulosic residues has been largely studied worldwide due to its considerable amount of potentially fermentable sugars [62]. The main structural components of Lignocellulosic Biomass (LCB), such as woods and agricultural residues, are cellulose, hemicelluloses and lignin. To produce ethanol by fermentation of complex polysaccharides (cellulose and hemicelluloses), different pretreatment methods including chemical and enzymatic hydrolysis are used to convert it into simple monosaccharides. Typically, lignocellulosic hydrolysates contain both pentose sugars, such as D-xylose and L-arabinose as well as hexose sugars. The main component of lignocellulosic hydrolysates is glucose (60-70%), and the second most abundant carbohydrate is D-xylose, consisting 30-40% of the cellulosic hydrolysates [63,64]. Thus, the development of economically feasible lignocellulosic ethanol production also depends on the utilization of xylose.

Hexoses are nicely fermented by *S. cerevisiae*, but not xylose [65]. Several microorganisms are able to ferment xylose, but none shows the interesting features to alcoholic fermentation as many as this yeast high ethanol productivity, multiple stress tolerance and resistance to inhibitory compounds present in the hydrolysate of lignocellulosic biomass [66]. Numerous studies have attempted different metabolic engineering strategies to overcome the restrictions of xylose metabolism and improve the xylose fermentation performance of *S. cerevisiae* [67,68].
Xylose-fermenting microorganisms use two different pathways to isomerize xylose into xylulose: the balanced-redox oxidoreductase and the isomerase pathway. The first one consists of two sequential enzymatic reactions: conversion of xylose into xylitol via Xylose Reductase (XR) followed by conversion of xylitol into xylulose via Xyitol Dehydrogenase (XDH); both enzymes use the same cofactor, avoiding redox unbalance. On the other hand, the Xylose Isomerase (XI) pathway, found in some bacteria and fungi, isomerizes directly xylose into xylulose without a cofactor requirement [69]. Both the oxidoreductase and the isomerase pathways have been favorably introduced into \textit{S. cerevisiae}, enabling recombinant strains to produce ethanol from xylose [70].

By comparing the xylose-fermenting abilities between a XI-expressing recombinant \textit{S. cerevisiae} strain and a XR-XDH-expressing strain, the first one has shown the lowest xyitol yield and the highest ethanol yield [71]. However, XI-expressing strains showed a much lower xylose fermentation rate. High ethanol yield and productivity were only achieved when XI expression was under strong promoters in multicopy plasmids, suggesting that high XI activities are necessary for efficient xylose fermentation. The cloning of XI from different microorganisms, \textit{Piromyces sp} [16], \textit{Clostridium phytofermentans} [72], \textit{Orpinomyces sp} [73], \textit{Prevotella ruminicola} [69], \textit{Burkholderia cenocepacia} [74], produced high XI activity in the recombinant yeast strains.

Some additional genetic modifications were necessary to enhance the performance of recombinant yeasts which isomerize xylose into xylulose. The overexpression of the endogenous XKS1 (codes for xylulokinase), the deletion of GRE3 (codes for a reductase able to convert xylose into xylitol using NADPH) together with the overexpression of all non-oxidative phosphate pentose pathway - PPP - enzymes, Tal1, Tk1, Rpe1, Rki1, improved growth on xylose and xylose fermentation in a strain carrying a bacterial XI [75] and also in a strain carrying \textit{Piromyces} XI [71]. Both the requirement of PPP activity for xylose metabolism and the increase in the activity to convert xylose to xylulose seems to be crucial for xylose fermentation in \textit{S. cerevisiae}. Subsequently, the relevance of high PPP activity in xylose-utilizing \textit{S. cerevisiae} was confirmed by metabolic flux analysis [76] as well as by microarray analysis [77].

Due to the absence of xylose-specific transporters, \textit{S. cerevisiae} has been engineered for xylose utilization. This microorganism assimilates xylose by facilitated diffusion mainly through non-specific hexose transporters encoded by HXT genes [78]. However, in ethanol producing strains, the xylose transport is limited in the presence of glucose and other pentose sugars through competitive inhibition during co-fermentation of glucose and xylose [79]. Xylose transporters derived from distinct xylose utilizing microorganisms have been cloned into yeast strains. Furthermore, the alteration of xylose interacting motif as well as the hexose transporters engineering have been also used to improve xylose transport [80].

\textit{S. cerevisiae} harboring \textit{XYL1} gene from \textit{S. stipitis} increased the xylose uptake about 7.9-folds on supplementing 20 g L\(^{-1}\) glucose [81]. In addition, the authors have shown an increased expression level of HXT4 and HXT7 genes, which encodes for Hxt4p and Hxt7p transporter proteins [81]. It has been also demonstrated that both genes designated GXF1 (glucose/xylose facilitator 1) and GXS1 (glucose/xylose symporter 1) have been expressed in \textit{S. cerevisiae} [82], and the recombinant Gxf1-expressing \textit{S. cerevisiae} strain has shown faster xylose uptake and ethanol production [83]. Recently, the incorporation of a transporter gene MGT05196 (xylose transport from \textit{Meyerozyma guilliermondii}) into \textit{S. cerevisiae}, showed a 26.3-fold higher growth as compared to wild-type strain [84]. Furthermore, it has been suggested that a mutation at N360F of MGT05196 enhances the D-xylose transport activities without any glucose-inhibition [84]. Thus, engineering specific xylose transporters without glucose inhibition, or overcoming the metabolic inhibition is essential for further improving the development of glucose-xylose co-fermentation strains for biomass refining.

According to some authors, glucose repression is a barrier for the use of xylose in lignocellulosic hydrolysates [85]. In contrast, Vilela et al. [86] demonstrated that the xylose consumption during fermentation of a glucose-xylose blend was improved when yeast cells expressing \textit{Burkholderia cenocepacia} XI were previously grown on glucose, as opposed to xylose alone [86]. Xylose-grown cells show increased expression of Mig1 repressed genes [87], as Mig1 is dephosphorylated and active only at high glycolytic rates [21] Thus, when growing on xylose, yeast cells switch the mode of metabolism from fermentation to respiratory, reducing the glycolytic flux which is detrimental for ethanol yield and productivity. Thus, the detrimental effect of glucose over xylose consumption seems not to be associated to catabolite repression.

Vilela et al. [86] used evolutionary engineering to improve xylose fermentation by the recombinant yeast expressing \textit{Burkholderia cenocepacia} XI, which involved sequential batch cultivation on xylose [86]. This strategy has been applied to increase the ethanol yield and productivity of xylose-fermenting recombinant yeast strains [30]. The improvement in xylose fermentation showed by the \textit{Burkholderia cenocepacia} XI recombinant strain submitted to evolutionary engineering was associated to the increase in the expression of HXT2 and TAL1 genes, which code for a low-affinity hexose transporter and transaldolase, respectively.

[86]. The use of xylose instead of glucose has several effects on the yeast metabolome that are specific to anaerobic consumption of xylose. For example, the reaction catalyzed by Tal1 is a rate-limiting step for the conversion of xylose into ethanol [88]. Hxt2 has the second highest transport capacity, taking up xylose at a rate of 8.74 g/h/g dry weight of cell at high sugar concentrations. Therefore, it should be expected a positive effect on xylose utilization under increased HXT2 and TAL1 expressions. Taken together, the results obtained by Vilela et al. [86] show that is necessary to understand more deeply the metabolic regulation of xylose-ethanol conversion yeast to increase the efficiency of fermentation [86]. Many works have tried to increase the flux of the xylose-ethanol
pathway in *Saccharomyces cerevisiae* by raising the supply of the enzymes. However, the activity of the enzymes depends on the environmental and intracellular conditions. This means that is necessary to understand how cells control their metabolism.

Currently, some of the most powerful tools for targeting metabolic changes to improve the xylose fermentation are functional genomics, including the transcriptome, proteome, metabolome and fluxome. These are emerging areas of future research for enhancing the rate and yield of ethanol production from xylose. Microarray technology as well as CRISPR/Cas9 technique have been also gradually used in xylose-metabolizing recombinant strains, for global expression studies and to improve xylose utilization pathways, respectively, providing important advances on xylose fermentation [87]. Although there still remain challenges in ethanol production from xylose using metabolically engineered *S. cerevisiae*, the combination of metabolic engineering with functional genomics analysis and/or evolutionary approaches may open novel avenues for developing strategies to an effective xylose fermentation as well as ethanol production.

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