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Review

Solving ethanol production problems with genetically modified yeast strains

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Abstract

The current world demand for bioethanol is increasing as a consequence of low fossil fuel availability and a growing number of ethanol/gasoline flex-fuel cars. In addition, countries in several parts of the world have agreed to reduce carbon dioxide emissions, and the use of ethanol as a fuel (which produces fewer pollutants than petroleum products) has been considered to be a good alternative to petroleum products. The ethanol that is produced in Brazil from the first-generation process is optimized and can be accomplished at low cost. However, because of the large volume of ethanol that is produced and traded each year, any small improvement in the process could represent a savings of billions dollars. Several Brazilian research programs are investing in sugarcane improvement, but little attention has been given to the improvement of yeast strains that participate in the first-generation process at present. The Brazilian ethanol production process uses sugarcane as a carbon source for the yeast *Saccharomyces cerevisiae*. Yeast is then grown at a high cellular density and high temperatures in large-capacity open tanks with cells recycle. All of these culture conditions compel the yeast to cope with several types of stress. Among the main stressors are high temperatures and high ethanol concentrations inside the fermentation tanks during alcohol production. Moreover, the competition between the desired yeast strains, which are inoculated at the beginning of the process, with contaminants such as wild type yeasts and bacteria, requires acid treatment to successfully recycle the cells. This review is focused on describing the problems and stressors within the Brazilian ethanol production system. It also highlights some genetic modifications that can help to circumvent these difficulties in yeast.

Key words: Brazilian ethanol production, yeast improvement, resistance to stress.

Introduction

Given the current environmental conditions and concern about the scarcity of fossil fuels, there is great interest in developing alternative energy sources with lower environmental impacts. Therefore, the use of bioethanol as a fuel has been widely encouraged. Depending on the material that is used (either sugarcane bagasse or corn) for bioethanol production, some processing is necessary, such as previous hydrolysis of the raw material, which can increase the cost of the final product. Because of the present demand for bioethanol expansion, efficient production is vital to the industrial process. One possible way to address this increased demand is through the genetic improvement of yeast strains that are used in the bioethanol production process.

Brazilian ethanol producers use the first-generation process because it has a high production yield at low costs, which occurs because sugarcane is already rich in sugars, the starting material does not need to be hydrolyzed and fermentation can be carried out in an open system. *S. cerevisiae* is an organism that preferentially ferments its carbon source, even in the presence of oxygen, and is widely used in Brazilian production processes (Amorim *et al.*, 2009).

Even with the high-yield production of current methods and the major technological advances in this area, every small improvement to the process increases the efficiency

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of carbon use and better utilizes the raw material (which is responsible for approximately 60% of the manufacturing cost) (Wyman and Hinam, 1990).

According to 2012 data from Conab (the national supply company) it is estimated that 302.2 million tons of sugarcane will be processed to produce 23.96 billion liters of bioethanol. This sum is 4.81% higher than the 2011/12 harvest. From this total, 14.21 billion liters correspond to hydrous ethanol (which is directly used as fuel) and 9.74 billion liters are made into the anhydrous form (which is mixed at a rate of 25% of the total volume in gasoline and for other industrial applications). According to these projections, anhydrous ethanol will increase by 7.44% in relation to hydrous ethanol and by approximately 3.08% compared with ethanol production from the previous years crop (Conab, 2012).

Many efforts in different fields have been carried out in relation to bioethanol expansion, such as substrate improvement, which is intended to increase the productivity and performance of microorganism fermentation (Matsutani *et al.*, 1992). The modification of selected genes can help yeast to cope with the stress that is caused by industrial processing (Zheng *et al.*, 2011). However, the creation of stress-resistant strains in the laboratory is not often transferable to industrial applications because of the open operational system that is used in Brazil for ethanol production.

Genes that were targeted for their alcohol sensitivity represent approximately 5-10% of genes encoded by the yeast genome. Some studies have shown that the loss of a specific gene leads to the creation of new strains that are tolerant to different types of stressors, including ethanol (Kim *et al.*, 2011; Yang *et al.*, 2011). More recently, 2 clones with high growth in 8% ethanol medium as well as 10 osmotolerant clones were identified in an SGKO (*Saccharomyces* Genome Knockout Collection) library-wide screening (Texeira *et al.*, 2009). Resistance verification is necessary following the identification of these target genes because the identification of stress-tolerant mutants can help investigators to understand the molecular basis of stress tolerance. However, these findings cannot guarantee the viability and productivity of the selected strains (Kong *et al.*, 2007). The setbacks faced within industrial production can be studied using modified strains to achieve better results and even to completely solve a particular problem. This review will focus on challenges that are encountered in the production of bioethanol and several modifications that are aimed at improving ethanol production by the Brazilian system.

Brazilian Industrial Fermentation Process

Over the past 30 years, the ethanol production process in Brazil (the design of which was based on a process that was used in France during the 30s) has been greatly improved. The process had a yield of 80% at the beginning of the ethanol production incentive program 20 years ago and has reached a current milestone of approximately 92%. This yield also includes the production of cell biomass, glycerol, succinate and malate during fermentation, whereas the other 8% is directed to cell metabolism. The Brazilian production process is characterized by the use of a must that is made by crushing sugarcane in large-capacity tanks (up to 3 million liters) at a high cellular density (10-15% w/v). The distilleries prefer fed-batch processes; continuous fermentation processes are used in 20%-30% of distilleries (Godoy *et al.*, 2008; Amorim *et al.*, 2010).

In Brazil, the outputs of fed-batch fermentation range from 400 to 2000 m^3 of ethanol per day. Typically, high yeast cell concentrations from 8-17% can achieve fermentation times of only 6-10 h with final ethanol concentrations of up to 11% v/v, which corresponds to an average ethanol yield of 91%. After each fermentation cycle, the yeast cells are separated, treated with dilute sulfuric acid to kill contaminating bacteria, and then recycled to start a new fermentation process. This sequence can be repeated up to 200 times and minimizes carbon consumption during yeast growth while providing very high ethanol productivity (Basso *et al.*, 2008). Fed-batch fermentation is one of the most common cultivation regimes in Brazil, mainly because *S. cerevisiae* present catabolic repression during the production process, *i.e.*, when high substrate concentrations inhibit specific metabolic processes including those that are related to the cell growth rate. For this reason, the microorganisms grow faster at low substrate concentrations. Nevertheless, the application of fed-batch cultivation to ethanolic fermentation still produces significant results by maintaining low substrate levels while ethanol accumulates in the medium. This regime for bioethanol production allows the achievement of high volumetric productivity (Sanchez and Cardona, 2008).

In a continuous fermentation system, the substrate is constantly fed into the reaction vessel, and a corresponding flow of fermented product broth is discharged to keep the reactor volume constant. Furthermore, a balance between the feed and the discharge is maintained for times that are intended to achieve steady-state operation with no changes in reactor conditions. In comparison to a batch reaction, this mode of operation offers reduced cleaning and vessel-filling down time and provides improved volumetric productivity that can translate into smaller reactor volumes and lower capital investments in addition to the ease of steady-state control (Godoy *et al.*, 2008). The design and development of continuous fermentation systems have allowed for the implementation of more cost-effective processes. Continuous processes have several advantages in comparison to conventional batch processes as a consequence of the reduced construction costs of bioreactors, lower maintenance and operation requirements, better process control, and higher productivity. The major drawback is that yeasts that are cultivated under anaerobic conditions over long time periods experience a diminished ability to synthesize etha-

nol. In addition, the substrate is not completely consumed, and yields are reduced at the high dilution rates that enable elevated productivity (Sanchez and Cardona, 2008).

In one study of the advantages and disadvantages of continuous and batch fermentation processes for 62 distilleries over a time span of 9 years (1998-2007), batch processes with recycled yeasts were shown to be less susceptible to bacterial contamination and the corresponding loss in productivity than continuous processes (Godoy *et al.*, 2008). *Lactobacillus* contaminations in particular are regarded as a major factor that can reduce ethanol yields and impair yeast centrifugation, and greater quantities of antibiotics are needed to address this issue in continuous processes. Additionally, slightly more sulfuric acid was consumed by continuous processes. However, continuous processes have the advantages of lower installation costs as a result of their smaller fermentor volumes and lesser heat exchanger demands. They also cost less as a result of their greater degree of automation in comparison to batch processing (Godoy *et al.*, 2008; Brethauer and Wyman, 2010).

Stress in the Brazilian Industrial Fermentation Process

The fermented must is centrifuged to separate the yeast from the wine. Subsequently, the wine is sent away for distillation, whereas the yeast cells are treated with sulfuric acid, which is intended to reduce bacterial contamination. After approximately 3 h, the cells are returned to the fermentation tanks to start a new fermentation cycle (Amorim *et al.*, 2011). Large amounts of the same biomass are recycled by the distilleries in a process that takes approximately 10 months overall, depending on parameters such as weather, climatic conditions, sugarcane quality, and product demand. The recycling of yeast cells is advantageous for industrial energy use and prevents the generation of excessive cellular mass. This process yields a lower energy cost related to cell growth that can instead be expended on sugar conversion, in which most of the material is converted to ethanol product (Amorim and Lopes, 2004). In comparison with other ethanol producers around the world, Brazil has a major advantage in terms of the low cost of brewer's yeast for this process. Although the production of bioethanol is a well-known process, the stress caused to the organisms during the process is not as familiar. Despite the operation of distilleries within approved conditions, the process begs for greater analysis because certain characteristic stress conditions occur in Brazilian distilleries.

Great biodiversity has been observed in industrial fermentation processes; each distillery owns a particular microbial population and shows succession of different strains (Basso *et al.*, 1993). Occasionally, some of these successive cells become persistent and prevalent, and strains with these characteristics deserve special attention because their behavior suggests higher competitiveness and tolerance to stress during industrial fermentation (Basso *et al.*, 2008). Most of the native yeast strains that are present during fermentation have some undesirable characteristics regardless of their dominant position and persistence, such as excessive foam production and high sedimentation rate (even during fermentation), long fermentation time and high residual sugar after fermentation. Strains that produce foam do not allow the full use of the fermentor's capacity and also consume more defoamers, thus increasing the costs of ethanol production. Furthermore, strains with high rates of sedimentation or flocculation may impair the centrifugation step of the process. Most strains that produce foam also have high flotation; both flocculation and flotation can reduce the contact between yeast and the substrate, increasing fermentation time and resulting in high residual sugar concentrations.

The manufacturing process that was designed for Brazilian plants works best with a homogeneous suspension of yeast during fermentation. There are few ethanol manufacturing plants in Brazil that work without a centrifugation step, and in these unusual cases, high rates of sedimentation are required. Some distilleries select only the current collection of their yeast population at the end of the fermentation season because these strains can be more tolerant of stress and can be pre-allocated to any sector of the fermenter. However, most yeast population selected does not have desirable production characteristics. Industrial fermentation strains are susceptible to contamination by bacteria and wild yeast (from *Saccharomyces* or other species), and these contaminants compete with the desired yeast strains to survive and consume the contents of the fermentors (Cabrini and Gallo, 1999).

The successive recycling of tons of yeast cells every day and the difficulty of sterilizing large volumes of water and juice allow microorganism contamination of the production process by competing with the inoculated yeast strains. These contaminating microorganisms have developed different strategies for survival and competition within the alcoholic fermentation processes. Gram-positive bacteria such as the *Lactobacillus* species are among the major contaminants during the alcoholic fermentation processes (Lopes *et al.*, 2004; Lucena *et al.*, 2010), whereas contamination by Gram-negative species is less frequent but is more difficult to control (Gallo, 1990; Lopes *et al.*, 2010). Bacterial populations are controlled with acid treatment, antibiotics, hop products and biocidal chemicals that do not affect the yeast cells (Amorim and Lopes, 2004). However, certain bacteria are more resistant to these compounds than others, making it difficult to control them during the recycling process. Furthermore, high bacteriological contamination can cause flocculation of yeast and can inhibit fermentation.

Yeast Strains Used in Brazil

Improvement in the first-generation process requires yeast strains that are able to survive in industrial conditions

and compete with wild yeast (Amorim *et al.*, 2010; Amorim and Lopes, 2005). Many studies have been developed around the world on the basis of the Brazilian system of production. The yeast strains CAT1 and PE2 (which were isolated from the Catanduva and Pedra plants, respectively) were selected after more than 20 years of industrial processes (by fed-batch and continuous fermentation) during a yeast selection program and have attracted the attention of researchers from around the world. The selection of these strains was performed by molecular karyotyping within the industry over a number of years (Basso *et al.*, 1993).

Brazilian distilleries have plenty of yeast strains on the market to use for industrial fermentation processes. Yeast that is used in baking or in the laboratory, as well as selected strains from other fermentation processes, does not usually survive more than one month at an industrial scale of operation because cell recycling, being rapidly replaced with wild yeasts (Basso *et al.*, 1993). The development of new strains for Brazilian distilleries may fail because they are based on samples that are unable to survive the recycling process after acid treatment, rapid fermentation cycles and competition with wild yeasts.

PE2 and CAT1 are clearly the most dominant yeast strains and have therefore been widely used in distillery fermentation processes for several years. These strains are diploid and heterothallic and they sporulate well. These strains are quickly able to resume meiosis, producing asci with three or four viable spores. Some studies suggest that the species has undergone evolutionary adaptations that cause chromosomal rearrangements that are already noticeable in industrial fermentation (Lopes, 2000; Lopes *et al.*, 2002). Despite the ability of yeast spores to mate with other individuals, there is little information about the chromosomal constitution and behavior of cells during meiosis and conjugation.

One point of great interest is the performance comparison between current yeast industrial strains and laboratory strains to identify what type of metabolic changes the industrial strains have suffered in exchange for their better performance. When introduced to an industrial environment, the baker's yeast and laboratory strains tend to fail to persist because they express genes that cause these strains to be unable to survive or adapt to the stressful conditions of alcoholic fermentation. Furthermore, these strains do not compete well against wild type yeasts and are quickly replaced by types that are more tolerant and adapted to their environment (Basso *et al.*, 1993; Lopes, 2010; Stambuk *et al.*, 2009).

Different researchers have investigated the special features of PE2 and CAT1 genetics (Argueso *et al.*, 2009; Stambuk *et al.*, 2009). To understand how gene expression is controlled in these organisms during the manufacturing process, it is necessary to characterize their genomes. Industrial yeast can serve as a background that receives foreign genes from recombinant DNA, which in turn provides the characteristic genetic trace that marks these specialized strains. This recombination can cause cells to lose their dominance or productivity-related qualities by affecting their gene balance, demanding a large quantity of research to develop an improved organism. For biotechnological purposes, it is necessary to understand the genetic constitution of *S. cerevisiae* wild yeasts that are found in the industrial fermentation process because they have complex features. DNA sequencing software, gene expression, proteomics, and metabolomics have become essential to understand how these strains are able to survive in conditions of very intense stress for hundreds of fermentation cycles. Combining knowledge of genetics, physiology, and industrial processes can introduce new features of interest into industrial yeast strains without affecting the ability of these new strains to survive successive cycles and the associated stress.

Obtaining strains that are tolerant to higher levels of stress is a strategy for ensuring the competitiveness of a given fermentation process. The production of ethanol during fermentation is limited by the inability of *S. cerevisiae* to grow in media containing high levels of alcohol (Fiedurek *et al.*, 2011). At the moment, there is great interest in the creation or identification of strains that can survive in high concentrations of ethanol while still maintaining their fermentative capacity (Teixeira *et al.*, 2009; Yoshikawa *et al.*, 2009). The use of more tolerant yeast strains is important for highly efficient ethanol production because alcohol concentration only continues to increase during fermentation to levels that can be harmful or lethal to cells. Yeasts that are tolerant to ethanolic stress may have some physiological differences such as intracellular accumulation of ergosterol, trehalose and proline (Kim *et al.*, 1996; Inoue *et al.*, 2000; Takagi *et al.*, 2005), which helps them to survive.

Genetic Modification for Yeast Improvement

Tolerance to ethanol

To develop ethanol-tolerant strains, different strategies were adopted to isolate new yeasts from natural resources or to make them resistant to stressors like, adaptive evolution. This strategy works on the principle that populations of cells can adapt to their environment over time by natural selection. Prior studies have used adaptive evolution to create mutant yeasts that are tolerant to various stressors (Stanley *et al.*, 2010), such as freeze-thawing (Takagi *et al.*, 1997), high temperatures (Wati *et al.*, 1996), high salt concentrations (Matsutani *et al.*, 1992) and high acetic acid concentrations (Aarnio *et al.*, 1991).

Other strategies, such as random chemical mutagenesis (in which a potent mutagenic agent is used to induce random modifications into the genetic material) (Mobini-Dehkordi *et al.*, 2008), genome shuffling (by subsequent rounds of sporulation and hybridization) (Shi *et al.*, 2008) or ultraviolet exposure (Yoshikawa *et al.*, 2009), have helped de-

velop the understanding of mechanisms that are used for cell protection and tolerance during alcohol production. It is currently possible to carry out the analysis of genomic DNA via microarray (Hirasawa *et al.*, 2007). Deletion mutant library (with single gene knockouts) screening and transposon-mediated mutant collections have been used to select genetically modified ethanol-resistant strains (Kim *et al.*, 2011; Teixeira *et al.*, 2009; Zheng *et al.*, 2011). A technology known as global transcription machinery engineering (gTME) can exploit a mutant library that encodes the TATA-binding protein in *S. cerevisiae* as a tool for creating ethanol-tolerant strains (Yang *et al.*, 2011).

A homozygous diploid yeast knockout collection was also screened to find yeast strains that grew faster than the wild type in medium containing 8% ethanol. The disruption of *URA7* or *GAL6* provided the mutant *S. cerevisiae* with increased resistance to different types of stressors, including ethanol. Additionally, these strains showed a higher glucose consumption rate at low temperatures than the wild type (Yazawa *et al.*, 2007).

Improved ethanol production

Some studies have yielded good results with the disruption of one or a group of genes that confer the ability of mutant strains to be more productive in ethanol than the wild type. To synthesize glycerol, which is a major by-product of ethanol production, the yeast would need to consume up to 4% of the sugar feedstock that is found in typical industrial ethanol processes. Ethanol production was improved by deleting the *GPD2* gene (glycerol 3-phosphate dehydrogenase) and over-expressing the *GLT1* gene (glutamate synthase) in *S. cerevisiae*. This improvement was achieved by minimizing glycerol formation and by increasing the conversion rate of NADH to NAD+ (Kong *et al.*, 2007). In another study, the gene *GPD1*, an NAD^+-dependent glycerol-3-phosphate dehydrogenase, was substituted by a non-phosphorylating $NADP^+$ GPD from *Bacillus cereus* in industrial ethanol-producing yeast. This resulting strain produced lower levels of glycerol and exhibited a higher ethanol yield (7.6 ± 0.1% than the wild type), but it failed to ferment on 25% glucose. In response, the authors overexpressed the 2 trehalose synthesis genes *TPS1* and *TPS2* in the mutant strain, and the resulting yeast displayed further reduced glycerol yields, which were indistinguishable from the maximum specific growth rate (μ_{max}) and fermentation ability of the wild type in anaerobic batch fermentations (Hirasawa *et al.*, 2007).

Improvement of heat and ethanol tolerance

The use of a mutant collection with non-essential deletions favors the selection of mutations that may be beneficial. A transposon-mediated mutant library was recently screened for heat and ethanol tolerance. 5 strains presented alcohol tolerance in medium containing up to 15% ethanol, and 2 of those were also tolerant to heat (42 °C). The simultaneous down-regulation of *SSK2* (a MAPKKK of the Hog1p pathway that is essential to yeast survival in high-osmolarity conditions) and *PPG1* (a putative serine/threonine protein phosphatase that is required for glycogen accumulation) that were produced by a transposon insertion in both promoter regions resulted in 1 of the heat and ethanol resistant strains. The other resistant strain was produced by a single gene disruption in the *PAM1* gene (which produces a protein of unknown function) (Kim *et al.*, 2011). The disruption of *MSN2* was related to ethanol tolerance. In association with Msn4p (which shares 41% of its identity with Msn2p), Msn2p regulates a global yeast response against stress that involves approximately 200 genes (Monteiro *et al.*, 2002). Single deletion mutants of *MSN2* or *MSN4* showed no obvious phenotype, but *msn2* and *msn4* double knockout mutants are hypersensitive to carbon starvation, osmotic and oxidative stresses as well as heat shock. The authors speculated that a single *MSN2* mutation could produce a higher ethanol resistance than the wild type because its absence leads to *MSN4* expression and, consequently, to the induction of *MSN4* target genes (Inoue *et al.*, 2000). Although *MSN2* and *MSN4* seem to be redundant, this result suggests that the specific genes that are regulated by the latter can be especially important in determining ethanol tolerance. Other overexpression studies showed that *MSN2* and *MSN4* genes caused yeast sensitivity to starvation and thermal stresses to be lower than usual (Estruch and Carlson, 1993). Both transcription factors cause similar changes in the lipid membrane (Okuyama and Saito, 1979) by regulating the genes that are responsible for encoding heat shock proteins (Ma and Liu, 2010). The overexpression of *MSN2* promotes ethanol tolerance and enhances the fermentation capacity of sake yeast strains (Watanabe *et al.*, 2009).

In conclusion, the use of genetically modified yeasts can help in the identification of genes that are responsible for higher stress tolerance and can possibly show which of them are involved in alcohol and heat sensitivity. This knowledge can guide investigators in the construction of improved mutants in CAT1 or PE2 backgrounds. There is a gap in the current literature in the description of yeast genes that are important to stress resistance during the cell recycling process, which is widely used in Brazil. This feature deserves further study to improve the ability of industrial yeast strains to defend themselves against stress within the fermentation process.

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