Chapter 9

# Phenotypic Characterization of Yeasts Aiming at Bioethanol Production

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**Abstract**

Worldwide, the production of bioethanol is derived through first-generation technology, where plants, vegetables, and cereals, that have high levels of sucrose, are fermented by yeast. Brazil, for the production of bioethanol from sugarcane, is among the world's leading producers. The process for bioethanol production is a complex that involves a variety of environmental factors, resulting in different phenotypic profiles of strain used. It has been evidenced that the interaction between environmental factors and microorganism can influence in the identification of different characteristics of *Saccharomyces cerevisiae*. Also, the bioethanol is developed by the second and third generations, and new yeast strains may also contribute to the feasibility of production. Successful performance of fermentation depends on the ability of the yeast to deal with a number of factors that occur during the fermentation, such as concentration of sugar, ethanol, nitrogen, pH, resistance to contaminants, stress protein, temperature change, and osmotic pressure.

**Keywords:** *Saccharomyces cerevisiae*, bioethanol, phenotypic, characterization, resistance

## 1. Introduction

In recent decades, recurrent crises in world oil have resulted in serious economic crises, leading to the search for alternative fuels [1]. In 1930, Brazil presented the first National Congress

on Industrial Applications of Alcohol that was aimed at establishing the infrastructure for the production and use of bioethanol. This led Brazil to start production in the early twentieth century, while other countries started the production of fuel from grains, using its potential for bioenergy production. The remainder of residues with high protein content is a source of nutrition in agriculture, as well as being a rich source of sustainability [2].

The three major world powers producing bioethanol are Brazil (sugarcane), the USA (corn), and China (wheat and corn), where Brazil is the largest producer through a direct source of sugar, as production by grains requires an additional step with the liquefaction and hydrolysis of the starch. Estimates indicate that around 85% of all bioethanol worldwide is the responsibility of Brazilian and North American production, as well as inferior productions in all parts of the continents, as shown in **Figure 1** [3].

In Brazil, the bioethanol had low volume of consumption compared to the use of conventional fuels, maintaining the Brazilian dependence on imported oil [4]. This made the national government launch the National Alcohol Program (ProÀlcool) in the 1970s, which established a new behavior for air quality and the development of technologies in the area of alternative sources of energy [5]. ProÀlcool represented the largest increase in bioethanol production, from 500 million liters at the beginning of the program to about 13 billion liters per year [6]. Since then, Brazil has been characterized as a potential producer of bioethanol, with a well-developed domestic consumption policy [6, 7].

Currently, Brazil has an estimated bioethanol production with the 2016–2017 crops of 33.2 billion liters [8] and has kept that figure since 1986. All the production comes from sugarcane, representing a large-scale technology characterized by the development of new cane varieties,

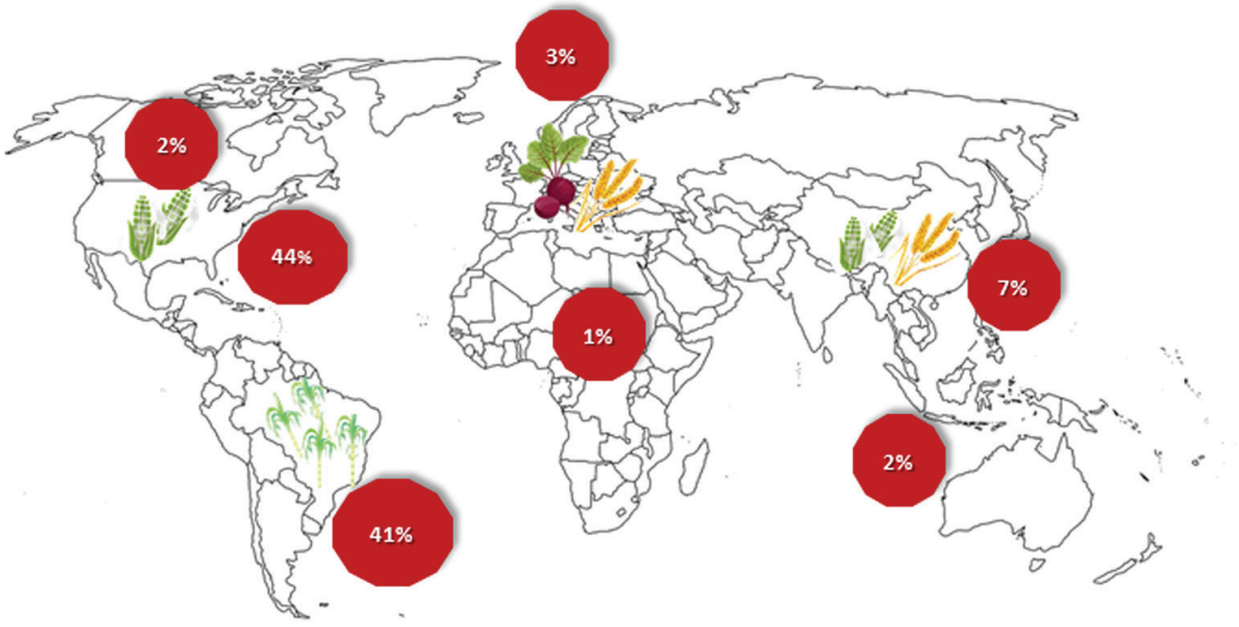


**Figure 1.** Global distribution of production and percentage of production per continent: Americas: South America—Brazil 43% (sugarcane); North America: USA 44% (corn) and Canada 2%; Europe 3% (vegetables and wheat); Africa 1% and Oceania 1%.

favorable climate, fertile soil, and advanced agricultural technologies [4, 6, 9]. The production of Brazilian bioethanol is derived from first-generation technologies, where a natural source of sugar from the sugarcane extraction, sucrose, is fermented by yeast with the primary product ethanol [10].

The sugarcane plant used for the Brazilian bioethanol production is derived the crossing from 637 species of the genus *Saccharum*, family *Poaceae*, *Andropogoneae* tribe, and native of hot temperate climate and with morphology characterized by stem and straw [11, 12]. The stem is the material from which the sugarcane juice is derived and is later used for the production of sugar and bioethanol. The bagasse is composed of all post-grind materials and the trash, characterized by the dry, green leaves of the plants, which serve as products of fermentation in second-generation processes for the formation of bioethanol [13].

The fermentation has been known since antiquity, being characterized as a biochemical and biological complex process, which has the objective of transforming sugar into ethanol (anhydrous and hydrated), carbonic gas, succinic acid, and volatile acids and esters [14].

The Brazilian fermentation process is differentiated and unique due to the fact that it is fed-batch in most states, being these short fermentation cycles and cell treatments with sulfuric acid [10]. This process uses cane juice as raw material, with a final product of 9–12% (v/v) and an efficiency of 90–92% [15]. The ratio of bioethanol produced to the amount of raw material used varies according to the amount of sugar present in the must, which consists of a mixture of molasses (sugar manufacturing residue), water, and sugarcane juice. The process starts with an action of invertase exoenzyme, in the process of breaking the sugar (sucrose, a disaccharide) into glucose and fructose (structural monosaccharaides), which are absorbed by facultative aerobic microorganisms, which under anaerobic conditions form the pyruvic acid cycle, the enzymes pyruvate with the help of decarboxylase and alcohol dehydrogenase, producing the bioethanol and its subproducts at the end of the fermentation [9].

The main key of the national fermentation process is that, at each end of the fermentation cycle, the yeasts are subjected to a centrifugation and sulfuric acid wash in order to minimize the risk of contamination [10]. At the end of this treatment, the cells are returned to the fermenters as a new inoculum for the subsequent cycle, this stage being repeated twice daily throughout the crop for 6–9 months, during the year, as shown in **Figure 2** [16].

The fermentation with grains (the USA and China) is rich in carbohydrates so it is essential to the stage of liquefaction and hydrolysis of this raw material, where the molecules of starches are broken down into fermentable sugars, and thus fermentation can occur, as shown in **Figure 3** [17]. One of the main characteristics of the grain fermentations, besides the additional stage of liquefaction and hydrolysis of the starch, is that the mills do not use recycled yeast cells, like the Brazilian mills, which is due to the fact that the whole concentration of residues and fermentable products is retained for distillation, decreasing the fermentation process when compared to the cell recycle process [18].

Significant changes are also observed when comparing the Brazilian and North American fermentation processes. In the fermentation of sugarcane, we have a lower concentration of solid residues, a concentration of larger yeast cells, and a much shorter time for bioethanol

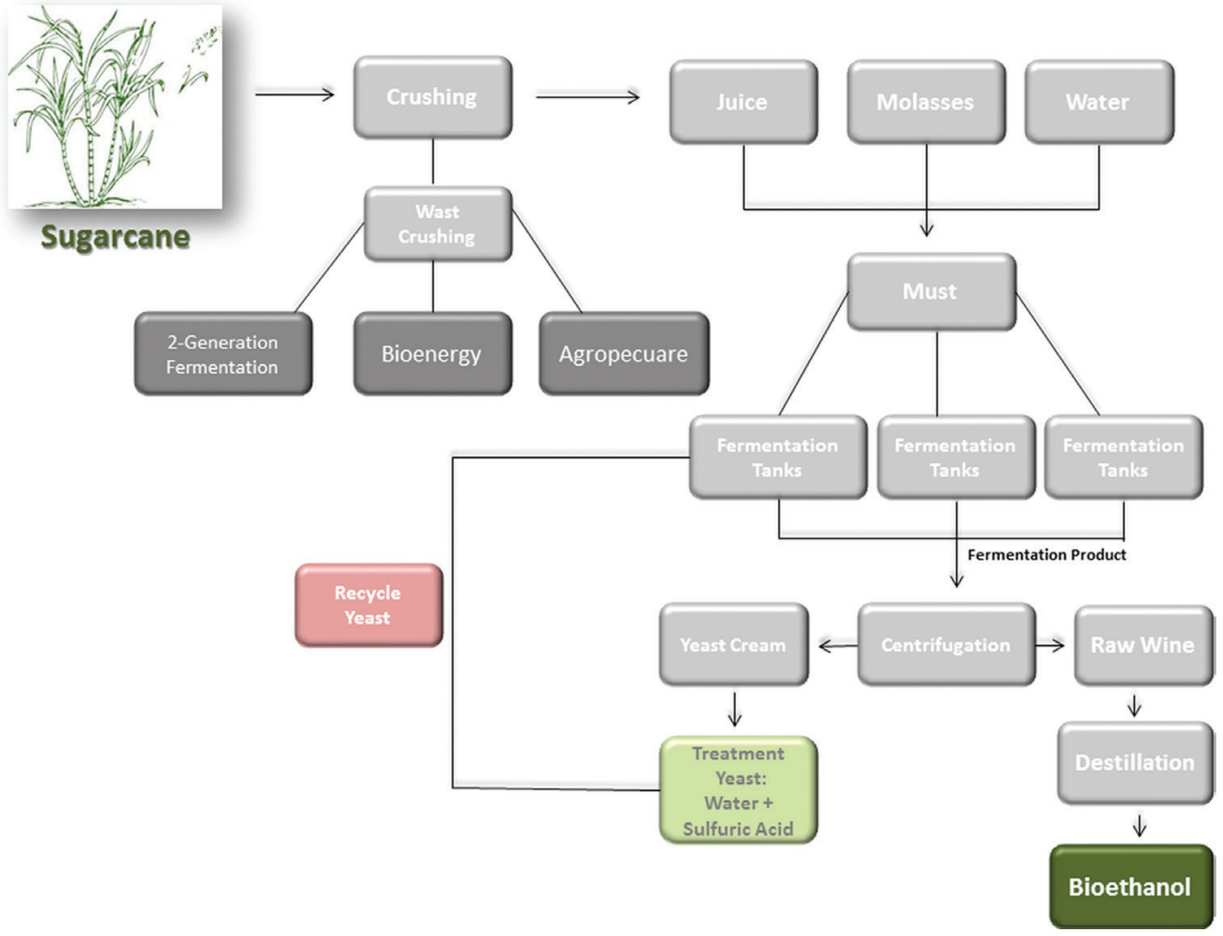


**Figure 2.** Simplified scheme of a fed-batch fermentation process with the recycling of yeast cells in Brazilian distillers by fermenting of sugarcane.

production, 6–12 h. The US process, that is derived for approximately 45–60 h, presents the advantages of a final concentration of bioethanol of 12–18%, against the 7–12% of the Brazilian process, and the raw material, that comes from corn plantations, lasts approximately one year, as opposed to the sugarcane harvest and its losses with rains that last around 200–240 day per year [19].

The main microorganisms used for the fermentation process are yeasts, such as *Saccharomyces* sp., *Schizosaccharomyces* sp., *Kluyveromyces* sp., among others [13]. Currently, the most used yeast in the sugar and alcohol sector, for fermentation processes in the production of bioethanol is the specie *Saccharomyces cerevisiae* [20]. The methodologies used for the identification of yeasts based on morphology, biochemical characteristics, and sexual reproduction require the evaluation of 70–90 tests to obtain the identification of species. Macroscopic and microscopy features may be the first method of identification of *S. cerevisiae* yeasts, as presented in **Figure 4** [21, 22].

The molecular techniques have been developed as alternatives to traditional techniques for the identification and characterization of yeasts, with the advantage of building an independent expression of the genes that allows quick and accurate identification of yeast species [23].

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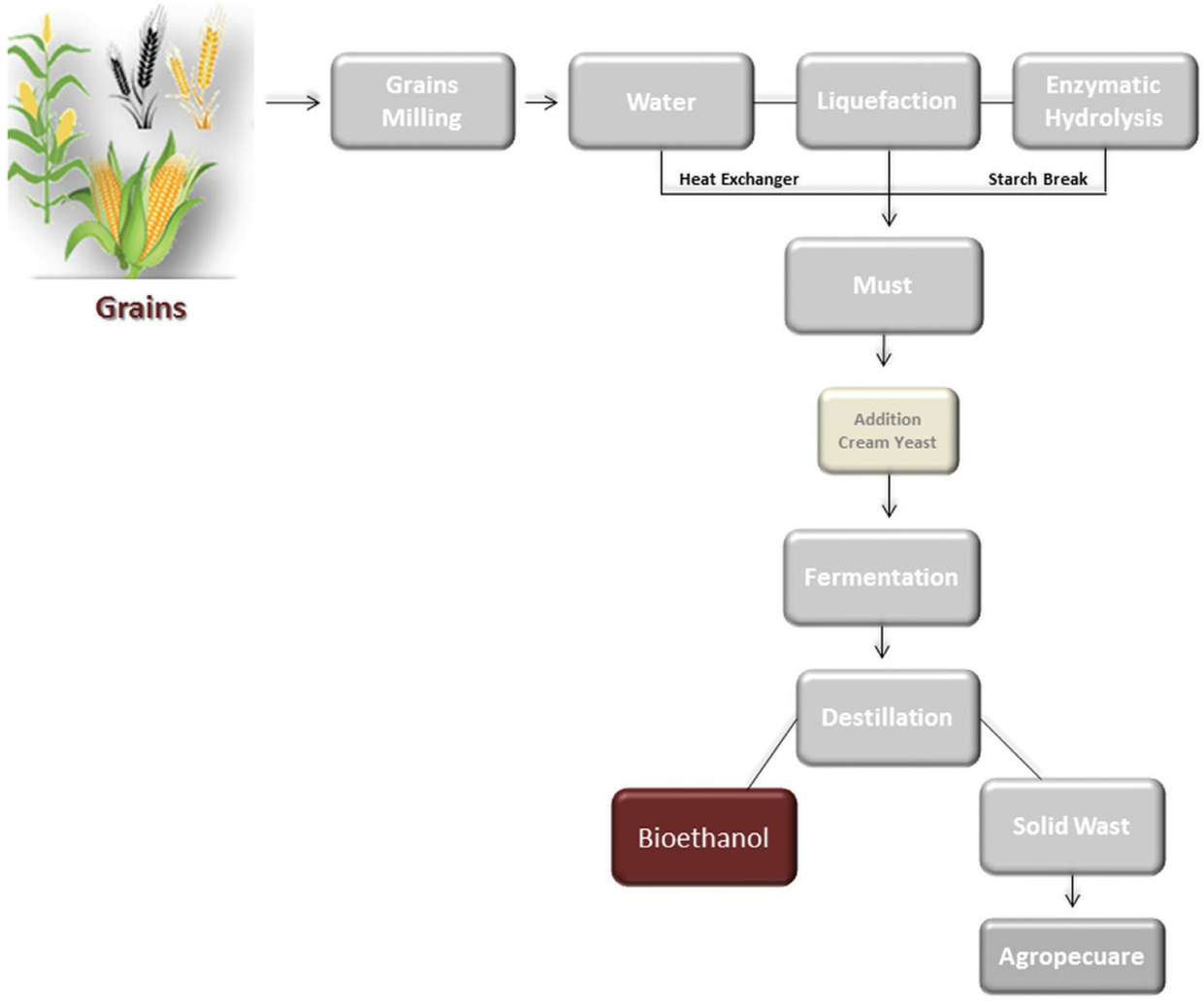


**Figure 3.** Simplified scheme of grains fermentation process with the liquefaction and hydrolysis in North American's and Chinese's distillers by fermenting of corns and wheat.

Due to the high mutation capacity of wild yeasts, molecular techniques for characterization and analysis of polymorphisms are being developed [24]. Genetic analyses of DNA, electrophoretic karyotyping, rRNA sequencing, rDNA restriction analysis, and polymerase chain reaction (PCR) and restriction fragment length polymorphism (RFLP) have been used as different tools to distinguish *Saccharomyces* sp. strains from strict sense group [25].

Studies of Melo Pereira [26] developed two new pairs of specific primers of the species, homologous to the HO gene of the species *Saccharomyces bayanus*, *S. cerevisiae*, and *Saccharomyces pastorianus*, offering a rapid method of PCR amplification, resulting in the correct identification of these species in less than 3 h. Guillamón [27] and Oliveira [28], by ribosomal DNA RFLP of ITS1, ITS2, and 5.8S identified different yeast species isolated from wine fermentation, and could also analyze the diversity of yeast species during spontaneous fermentation.

*S. cerevisiae* is characterized by being yeast with growth in media containing simple sugars and disaccharides, high genetic transformations, and qualities of high resistance to adverse

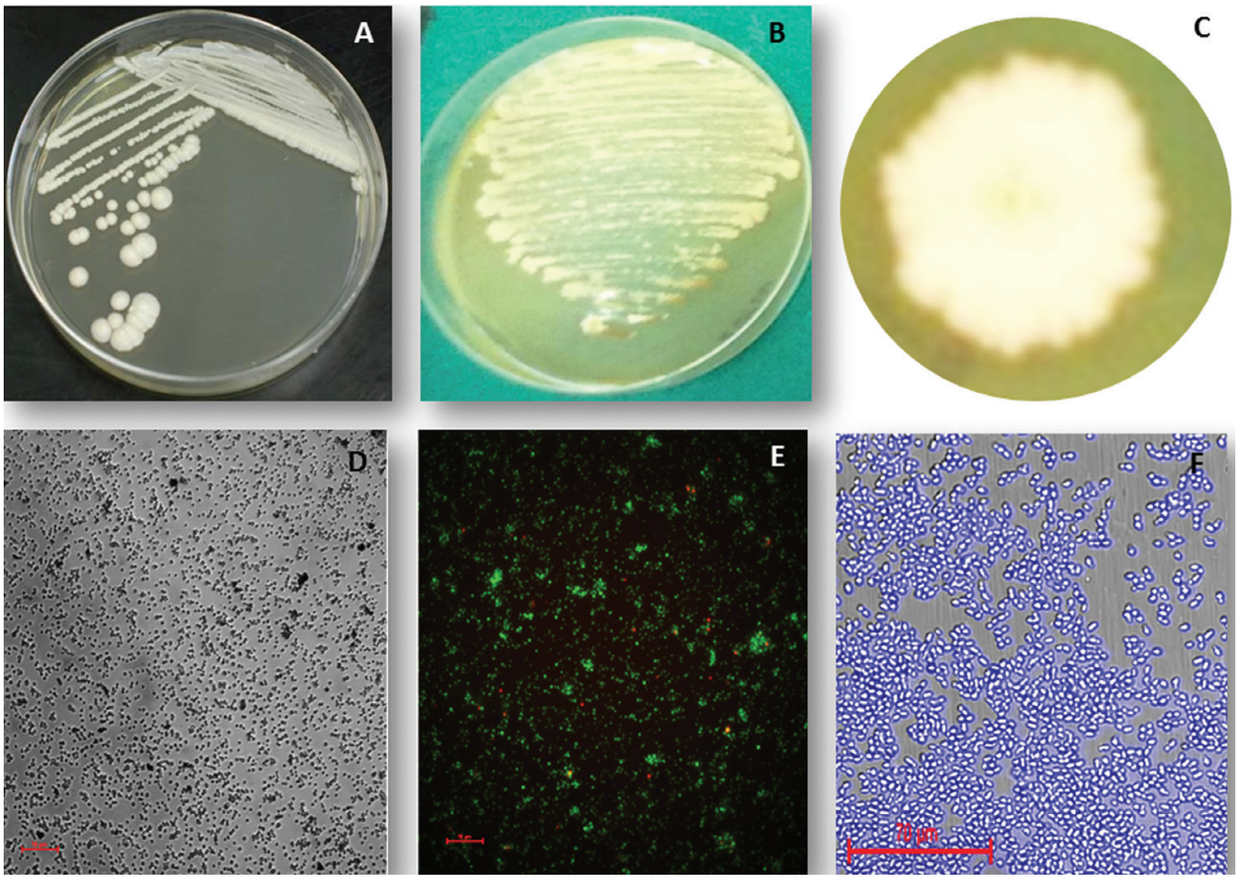


**Figure 4.** Macroscopic and microscopy of yeasts Saccharomyces cerevisiae. Macroscopies (A), (B), and (C) of PE-2 isolates, grown in YEPD (yeast extract-peptone-dextrose growth medium) solid with creamy and yellowish-white culture characteristics. Microscopies: (D) clear field microscopy of PE-2 isolate; (E) PE-2 isolate with FITC (fluorescein isothiocyanate fluoroforo) and Propidium Iodide cell tags; (F) PE-2 isolate with Calcofluor cellular target; Microscopy presence of oval yeasts with budding presence, with size of approximately 4–8 μm. Microscopies were performed in IN Cell Analyzer, objective of 20× and diameter 70 μm.

conditions of the growth medium, offering a primordial role in the processes of fermentation [29]. Some strains of *S. cerevisiae* have the capacity to be highly productive, dominating the entire fermentation process during the harvest period, allowing efficient and stable fermentations, which result in lower costs and higher fermentation performance (high production capacity of ethanol), and high viability throughout the process [30].

Studies indicate that *S. cerevisiae* is adaptable to different environments, revealing to be a rich source of phenotypic profiles in the *Saccharomyces* sp. species evolution [31, 32]. It has recently been shown that the interaction between environmental factors and organism may influence the identification of different specific characteristics of *S. cerevisiae* [33, 34]. *S. cerevisiae* is widely used and cultivated in industrial fermentation, due to the high capacity of the yeast adaptations to the variable conditions of the environment, such as sugar and ethanol concentrations, pH, oxygen concentrations, resistance to contaminants, salt stress, protein stress, temperature changes, and osmotic pressure [35].

According to Gao et al. [36], using thermophilic strains is interesting in the processes that involve simultaneous saccharification and fermentation (SSF), as this process may reach from

45 to 50°C, resulting in a greater bioethanol production. This occurs because the yeast suffers less damage with the temperature increase and there is a lower chance of microbial contaminations. It is therefore desirable that the thermostable yeast fermentation occurs at the optimum temperature of the enzyme, maximizing the ethanol production process. Thus, the increase in thermotolerance in yeast results in cost production, increases yield in the ethanol production with simultaneous saccharification and fermentation (SSF) system and reduces the possibility of contamination.

However, it is important to know the fermentative yeasts for the control and monitoring of alcoholic fermentation, especially in search of selected characteristics of dominance and resistance to bioethanol yeast production. The objective of this chapter is to distinguish the main phenotypic characteristics of *S. cerevisiae* yeasts in the alcoholic fermentation, for a possible selection of new strains with differentiated phenotypic characteristics, resistant and ideal for the production of bioethanol.

## 2. Phenotypic parameters of *S. cerevisiae* in alcoholic fermentation

### 2.1. Flocculation test

Cell flocculation of yeast strains such as *S. cerevisiae* is called cell aggregation and sedimentation in liquid media [37]. Cells have the characteristics of agglomeration at the end of each fermentation process, which makes it an interesting and divergent phenomenon in the industry, as shown in **Figure 5** [38]. Studies point out some divergences in flocculation, which can be a phenomenon of cooperative protection mechanism found in cells during adverse factors in the fermentation cycle [39]. It also facilitates the separation of yeasts at the end of the fermentation by sedimentation, thus helping the collection, centrifugation, and cellular treatment, contributing to the new inoculum stage, for a next cycle in the fermenter [40].

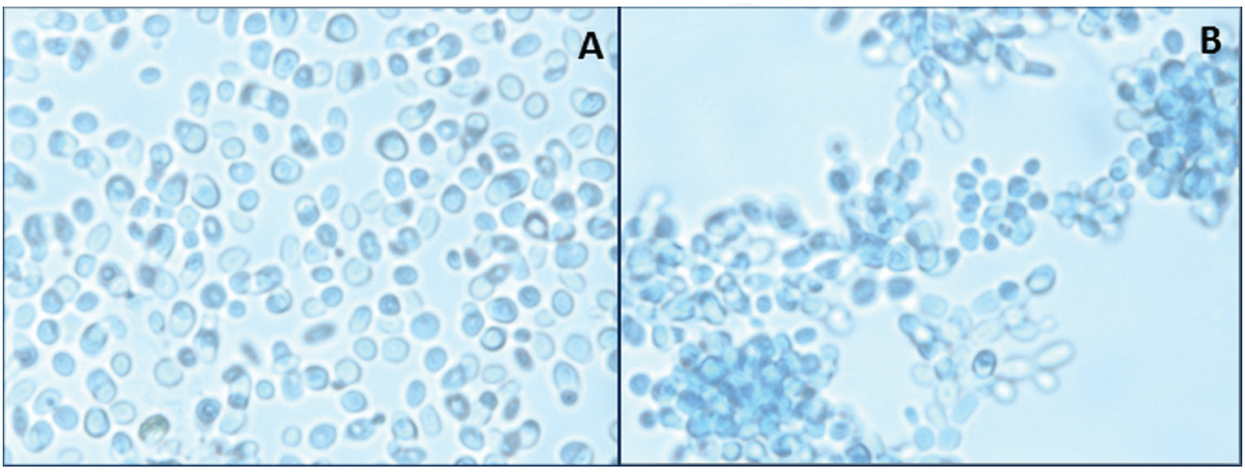


**Figure 5.** Characterization of aggregative power of *Saccharomyces cerevisiae* yeast cells, grown in YEPD liquid medium and stained with lactophenol. (A) Cells in normal condition without aggregative power. (B) Cells in condition of cellular aggregation.

The principle of cell adhesion is initiated through the recognition of mannose chains, located on cell surfaces, by lectin-like proteins, and agitation is necessary for the beginning of flocculation [41]. Two hypotheses for flocculation are well established: (I) sensitively to proteinases; (II) inhibited by saccharides, suggesting the existence of a protein that recognizes these sugars [42].

These two hypotheses classify two flocculation groups in yeasts that are distinguished by the inhibition of sugar: the first group, called New Flo phenotype, characterized by the inhibition of mannose, glucose, maltose, and sucrose with the exception of galactose; and the second group, the Flo1, is inhibited by mannose, but not by glucose, maltose, sucrose, and galactose, and its action is normally bound to a gene [43].

It is believed that these two distinct phenotypes are caused by two different proteins of the lectin type. Furthermore, the physical-chemical interaction in the cells surface may be involved in the aggregation process, where there is a correlation between flocculation and electrophoretic mobility of yeast cells on certain stress conditions. Other studies reported the correlation of hydrophobicity in the process of flocculation [44].

Another hypothesis for flocculation is the action of a dominant gene family (FLO1, FLO5, FLO8, FLO9, FLO10, and FLO11), where they encode a yeast cell wall protein that acts directly on the cell aggregation [45]. The proteins encoded by these FLO genes share a cellular/modular organization in three domains: an amino-terminal responsible for carbohydrate binding, a central domain, and a carboxyl-terminal domain containing a glycosylphosphatidylinositol anchor sequence [46]. However, the central domain contains tandem repeat regions of DNA sequence that can drive recombination reactions within and between FLO genes, resulting in new generations of FLO alleles, thus conferring yeast cells a wide diversity in the flocculation phenomenon [47].

### 2.2. Sensitivity test temperature and ethanol

The environmental adversities occurring in a fermentation cycle, such as the decrease of nutrients by sugar consumption, temperature changes, pH changes, risk of contamination, phenolic compounds, and the concentration of ethanol by its own production occurs in different forms and some of them were completely studied [48]. An understanding of the cellular mechanisms of protection to the multiphysical and chemical stresses that the yeast undergoes during fermentation cycle is fundamental for the selection of ideal yeast [49].

Temperature elevations result in reduced fermentation efficiency in *S. cerevisiae*, due to the high fluidity in the membranes, caused by the altered composition of fatty acids in the adverse response [50]. As one of the stress factors known in the fermentative cycle in yeast, temperature change restricts ethanol production and induces the accumulation of proteins bound to tolerance stress [51].

In the first-generation fermentation cycle, yeasts require a temperature of 30°C, whereas, in the production of second-generation bioethanol, where cellulose enzymes start the process by saccharification, yeasts require a higher temperature of 45–50°C [52]. The efficacy of the

fermentation is decreased at high temperatures, because it causes damage to the yeast cell, such as the rupture of the protein structure or the loss of function, thus preventing cell proliferation, decreasing viability during the process, and leading to cell death [53]. This temperature control in the fermentative cycles is a problem for the plants in tropical countries, where the ambient temperature is already naturally high and cooling systems are necessary for the total control of this temperature [54].

In the bioethanol production, the process temperature must be stabilized at around 30°C (the cell growth temperature), which is reaching 40°C [55, 56]. Thus, thermotolerant yeast strains may be a promising approach to a profitable fermentation process, as is the case of simultaneous saccharification and fermentation that requires high temperatures to increase ethanol yield [36].

Osmotolerance can be an important factor in the production of ethanol for its adaptation strategy employed in all cell types by accumulating compatible solutes (sulfite), resulting in a decrease in the potential of intracellular water [57]. As sulphite and sulfite-generating compounds have long been used as antimicrobial agents in alcoholic fermentation, tolerance to sulfite in yeast is another desired characteristic for the production of bioethanol from sugarcane juice [58].

The high levels of ethanol in the fermentation medium are considered as negative parameters in the process conditions, because at the same time that the production is essential, the accumulation of ethanol by this production generates an acidification of the medium, leading to irreversible damages in the yeast membrane, thereby decreasing cell viability [59].

The true physiological and ecological relevance of ethanol tolerance in *S. cerevisiae* is its ability to generate mechanisms that protect the cell from chemical and physical damage at high levels of ethanol [60]; this is usually observed in a typical fermentation environment, where there is a large amount of sugars, leading later to ethanol production [30]. This stage generally occurs by stationary phase cells and its tolerance to the ethanol produced is only controlled by the integrity of the yeast membrane in contact with the ethanol accumulation, which is composed of chitin, glucans, glycoproteins, fatty acids, and ergosterol [61].

However, *S. cerevisiae* is resistant to ethanolic stress for its capacity of modifying the conformation of its membrane in the increase of fatty acids and ergosterol when coming in contact with the adverse environment, thus neutralizing the damages caused, mainly in relation to its viability [62, 63]. The accumulation of ethanol can also affect the structural compliance of the cellular proteins causing the inefficiency of its actions, such as the decrease of the activity of glycolytic enzymes: pyruvate kinase and hexokinase, besides altering the absorption of glucose, maltose, and amino acid. In some cases, there may occur cellular extravasation of essential cellular components [64].

In industrial fermentations, a high capacity of production is observed by the accumulation of ethanol in the medium, indicating a positive assimilation of residual sugar, which is measured by the visualization of cellular proliferation in the presence of the gradual levels of ethanol produced during the fermentative process [65]. Tolerance and ethanol characteristics of the main industrial strains of *S. cerevisiae* studied are described in **Table 1** [30, 66–69].

| Strains | Group | Origin | Feedstocks | Temperature tolerance (°C) | Ethanol tolerance (%) |
|---|---|---|---|---|---|
| ZTW1 | Industrial (fuel ethanol) | China | Grains | 55 | 18 |
| YJ5329 | Industrial (fuel ethanol) | China | Grains | 55 | 18 |
| PE-2 | Industrial (fuel ethanol) | Brazil | Sugarcane | 40 | 15 |
| CAT-1 | Industrial (fuel ethanol) | Brazil | Sugarcane | 40 | 15 |
| AT-3 | Industrial (fuel ethanol) | USA | Grains | 40 | 14 |
| ErOh red | Industrial (fuel ethanol) | USA | Grains | 40 | 15 |

**Table 1.** Characteristics of temperature tolerance and ethanol for major industrial strains worldwide used for the production of bioethanol.

Although this assay is routinely used in industries as large-scale screenings, its actual importance in ethanol resistance in yeast is not elucidated, due to divergent of actions that this process can cause, for example, the negative side acting in the decrease of the cell viability, and positive the increase in resistance to contaminating microorganisms in the fermentation process [70].

The metabolic pathways correlated to the expression of genes responsive to high levels of heat stress and ethanol stress include heat shock proteins (HSPs) and also metabolic enzymes such as trehalose, which is directly involved in tolerance in *S. cerevisiae* [71]. HSPs play a role in folding and refolding, transport, and degradation of intracellular proteins, triggered by stress in fermentation process and located in the cytoplasm, nucleus, and mitochondria, acting immediately in response to an accumulation of denatured proteins, activating the transcription factors of thermal shock (HSF), and leading to a positive regulation of thermotolerance gene expression [72].

The interactions of multiple genes at loci for cellular functions under heat and ethanol stresses are essential [73]. HSPs are known as chaperones ensuring the functional and structural conformation of the yeast, on the action of genes such as SSA1, SSA2, SSA3, and SSA4 which are expressed together with the HSP genes HSP12, HSP26, HSP30, HSP31, and HSP150 which were also found active at high stress levels [74] and interactions between chaperones of different types are widely encountered [75].

However, the inference of several chaperones shows an effective activity in neutralizing the stress, with the activation of the functional chaperones specific to more complex structures in the yeast cell walls [76], which have as a main function to repair of these denatured proteins to maintain cell viability [77].

In addition to serving as chaperones, HSPs have numerous other functions, for example, Hsp30p is characterized as a hydrophobic plasma membrane protein that acts on the regulation of $H^+$-ATPase, Hsp31p, and Hsp32p functions as hydrolases and peptidase, and Hsp150 is characterized as a protein in supporting the cell wall stability and remodeling [78]. HSPs and chaperone-mediated genomic regulation are also linked to glucose metabolism, which

are indispensable tools for stress tolerance in yeast metabolism, especially with storage of carbohydrates, such as trehalose [79].

Trehalose is a compound that acts to prevent the influx of excess salts resulting in irreversible dehydration of cells; therefore, yeasts are capable of accumulating trehalose up to 15% in a stress environment [80]. The trehalose acts by reducing the permeability of the membrane thereby rendering it hydrophobic, due to some regulatory genes such as TPS1, TPS2 and, TSL1, as well as acting in the remodeling of proteins under stress conditions [81]. Cells incapable of accumulating trehalose presented depreciated growth, leading to a significant decrease in cell viability during fermentation stresses [82].

### 2.3. Assimilation of sugars

Sugarcane juice is one of the main means used in the production of bioethanol, which is derived from the break of fermentable sugars such as sucrose, glucose, and fructose in contact with fermenting microorganisms such as yeast *S. cerevisiae* [83]. Yeast consumes the sugars in the medium in a complex and highly regulated manner, the principle of fermentation, where the sucrose is consumed first, followed by glucose and fructose, and finally maltose, this assimilation of sugars can occur simultaneously between the breaks of sugars, which is the standard process for sequential uptake of the glucose repression pathways or the catabolite repression pathway [84].

Glucose and sucrose may trigger beneficial effects on cells, including stimulation of cell proliferation, mobilization of storage compounds such as glycogen and trehalose, as well as decreased resistance to cell stress [85]. In contrast, negative impacts due to lack of glucose in the process can lead to several problems such as decreased or blocked fermentations, instability of cellular viability and low ethanol production [86], where the break of sugars, sucrose into simple sugars (glucose) occurs by an intracellular enzyme known as invertase, located in wall the yeast industries [87].

Microorganisms that possess the ability to assimilate the highest amount of sugars are indicated for the production of bioethanol, examples are shown in **Table 2** characterizing the main strains of *S. cerevisiae* worldwide used in industries for the production of bioethanol [30, 66–69].

| Strains | Group | Origin | Feedstocks | Assimilation sugar (%) | Production bioethanol (%) |
|---|---|---|---|---|---|
| ZTW1 | Industrial (fuel ethanol) | China | Grains | 65 | 28 |
| YJ5329 | Industrial (fuel ethanol) | China | Grains | 60 | 33 |
| PE-2 | Industrial (fuel ethanol) | Brazil | Sugarcane | 51 | 22 |
| CAT-1 | Industrial (fuel ethanol) | Brazil | Sugarcane | 52 | 26 |
| AT-3 | Industrial (fuel ethanol) | USA | Grains | 42 | 18 |
| ErOh red | Industrial (fuel ethanol) | USA | Grains | 75 | 30 |

**Table 2.** Characteristics of the assimilation of residual sugars and ethanol production for large industrial strains of *Saccharomyces cerevisiae* yeasts used in the production of bioethanol.

The assimilation of sugars in the fermentation process is not exclusively the fermentation of sugarcane. Currently, new technologies are available to produce ethanol from vegetables such as potatoes, cassava, beets, cereals such as corn, and there are also studies showing the production of green bioethanol in algae fermentations [88].

This type of fermentation is due to the breakdown of starch, carried out by the action of the enzyme glucoamylase, acting directly on the conversion of starch to glucose, by breaking the successive bonds of the nonreducing end of the glucose finally producing straight chains [89]. The process of producing ethanol from starch involves two main steps: enzymatic hydrolysis as the main step and habitual fermentation as the second step [90].

### 2.4. Second-generation bioethanol

All adverse parameters studied for the first-generation fermentation process have been highly researched to reach an ideal model of production of second-generation bioethanol, which is characterized by being profitable and environmentally sustainable [91].

Second-generation bioethanol production starts from the lysis of the raw material (sugarcane bagasse, vinasse, and residues from the milling of grains). The main step is characterized by a pretreatment where the breakdown of the cellulose-hemicellulose-lignin complex allows the production of fermentable sugar levels for a subsequent fermentation, demonstrated in **Figure 6** [92].

The hydrothermal and lime pretreatments are the most used, known for making the method more effective in preparing the biomass bioconversion step [93], a strong advantage for the sugarcane bagasse. They can be carried out under conditions of low temperature and pressure, resulting in lower sugar degradation, whereas in the saccharification the pretreatment is observed with high temperatures and difficult breaks of carbohydrate chains, resulting in a lower amount of sugars [94].

The fermentation of lignocellulose hydrolysates for bioethanol production presents two main problems: first, the fermentation of xylose that requires a low and controlled oxygenation; second, the removal of microbial inhibitors, which can contaminate the process [95]. Furthermore, these yeasts present a certain tolerance limited to ethanol [96].

Genetic manipulation in the metabolism of xylose in yeast fermentation has advanced and pioneering studies on glucose transporters that mediate xylose uptake, allyl-xylitol-reductase genes, xylitol dehydrogenase and xylulokinase have been expressed, which allows a better assimilation and fermentation of xylose [97].

The main concern in this step is that the balanced supply of NADP (enzyme nicotinamide adenine dinucleotide phosphate) and NADPH (enzyme nicotinamide adenine dinucleotide phosphate oxidase) has to be constant to avoid the production of xylitol. The path is the reduction of NADPH production by blocking the oxidative pentose phosphate cycle in xylose assimilation [98]. Cellulose hydrolysates present different inhibitors from lignin derivatives and sugar degradation, resulting in high amounts of acetic acid, intrinsically necessary for the deconstruction of biomass [99].

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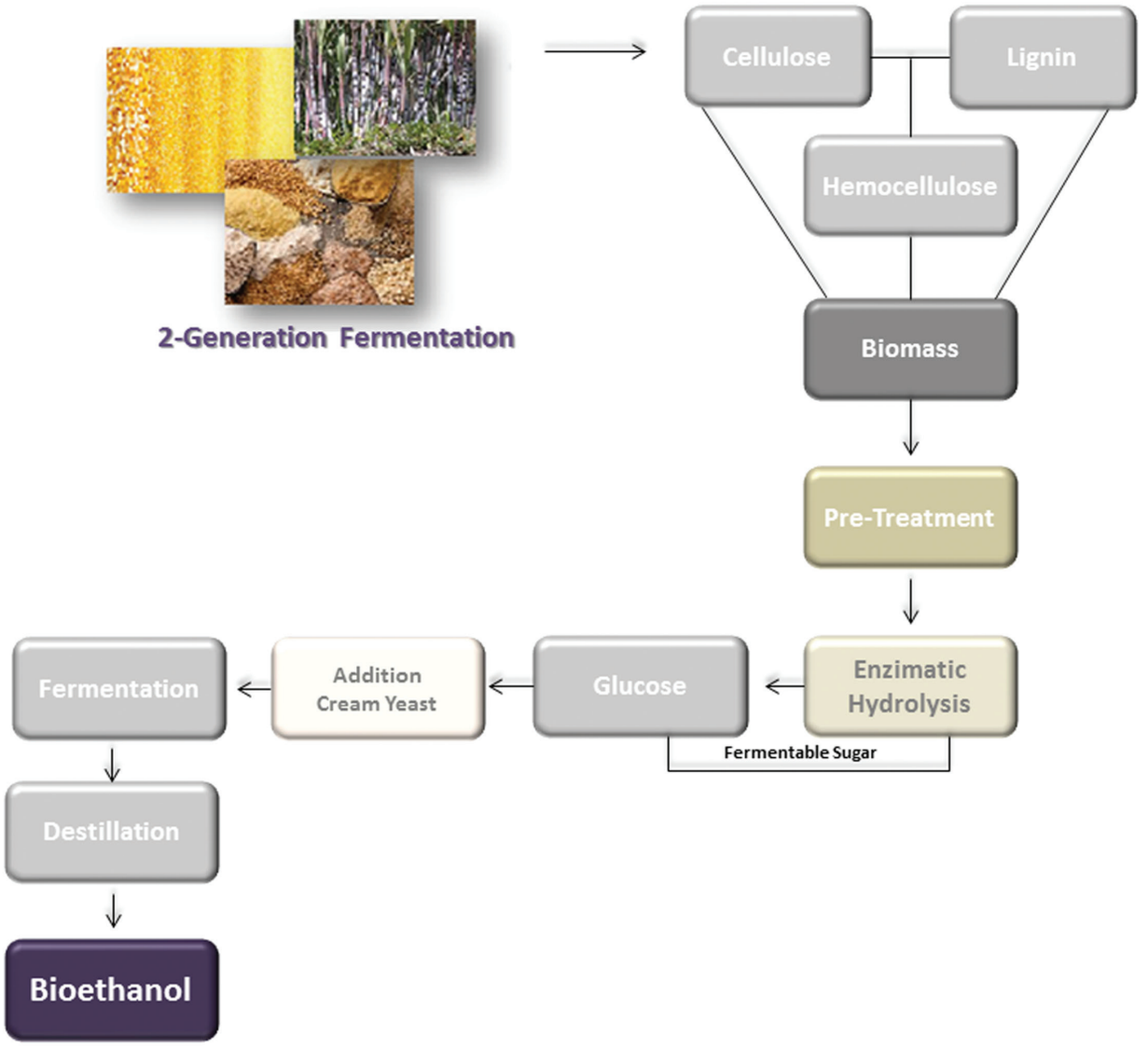


**Figure 6.** Simplified scheme of second-generation fermentation process with steps of biomass and hydrolysis for bioethanol production worldwide.

All adversities of typical fermentation first generation associated with pH, temperature, elevation of ethanol concentrations, and temperature among other stress factors present in large-scale fermentations are seen together in the adversity challenges of second-generation fermentation [100].

### 2.5. Advances and perspectives

First-generation fermentation over the years has mainly been used for large-scale industrial models. Although it is a well-established process, it is not definitively elucidated. Changes can be seen with each new process initiated presented for fundamental parameters and the behavior of the yeasts used.

*S. cerevisiae* has the characteristic of being adaptable to any environment, which leads to numerous behavioral responses during fermentation. As for each new cycle, changes are inevitable and checking all parameters of fermentation are of extreme importance for the success of the fermentation.

Flocculation is a divergent parameter, although it can have many advantages as a phenomenon of cellular protection to several stressors and contaminations in a process, it also presents disadvantages such as low yield in fermentation of fermenting tanks by their decanting. The question whether this phenomenon is beneficial or detrimental on flocculation is still uncertain; however, it is well-known and elucidated in its morphology or molecular action in yeast cells, and it contributes to the improvement of bioethanol production in the world industry.

For an alcoholic fermentation to be efficient, it is necessary and indispensable to know what happens throughout the process, the main steps and degrees that microorganisms go through for hours and days in order to remain viable and productive. For this reason, the study and knowledge of the two main parameters stress of fermentation (heat and ethanol) is of paramount importance for any beginning of the process, whether in small scale, as in laboratories, or large scale, as in industrial productions. The behavioral responses of the fermentation are measured through these parameters that are observed at all times, always aiming the improvement for the process.

The main step for a virtuous bioethanol production is the ability of the microorganism to breakdown the sugars and thus assimilate them to ferment. This detailed step has to be well studied so that there is no damage throughout the process, especially at industrial scales, so that both, a sufficient amount of microorganism concomitantly and adequate amount of sugars are essential to the start of the production.

Looking at the current scenario, the first-generation processes were modernized and studies and improvements resulted in second-generation fermentation, which aims to take advantage of all remaining residues and reaches to more sustainable processes. These processes are taking strides and improvements are being seen at all times to reach the ideal process.

In view of this profile, the search for yeasts with more robust characteristics in industrial lines is essential, and different strategies involving adaptation and functionality are highlighted by genetic engineering research. Advances in the area of a process and ideal yeast are positive, but the journey is still far from reaching perfection. The secrets and mysteries of fermentation are innumerable, but research is constantly revolutionizing and little by little these are being unraveled and the beginning of everything is the understanding of all the steps and all its parameters.

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