Review

Ethanol Synthesis by *Saccharomyces cerevisiae*: Summary, Discovery, and Prospects

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Abstract: *Saccharomyces cerevisiae* (*S. cerevisiae*) ethanol fermentation has left an indelible trace in human history. It is regarded as the original beverage synthesis, and has developed into an essential energy supply for mankind today. However, the history of ethanol synthesis using *S. cerevisiae*, including the mechanism analysis process, has not been carefully and succinctly reported. In this paper, we review the history of ethanol synthesis using *S. cerevisiae*. In addition, we also review the substrates that drive *S. cerevisiae* ethanol synthesis, including the utilization of glucose, sucrose, starch and molasses, in order to summarize the current development status and to contribute to the further improvement of this field.
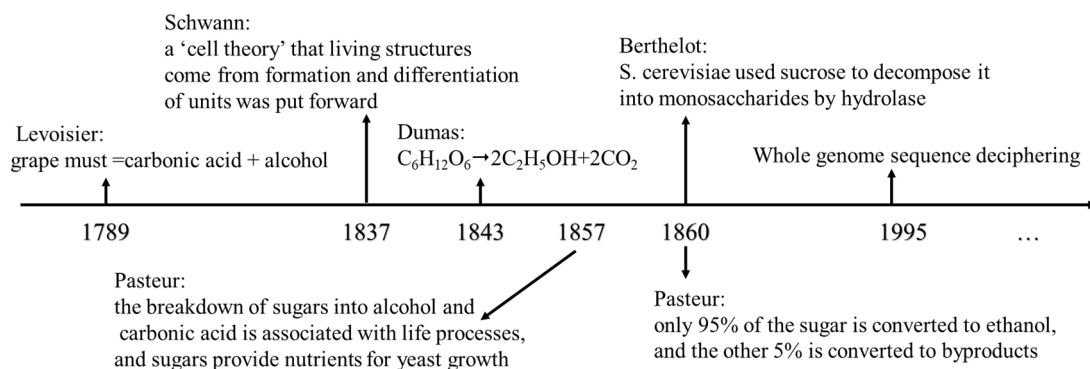
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1. Introduction

Ethanol has been used as a human beverage for thousands of years, and it has been recorded in the development history of the ancient civilizations of the world. The early use of ethanol may be attributed to its analgesic, disinfection, and spiritual effects on weddings, funerals, military expeditions, and the healing of patients. With the deepening of people's understanding of microorganisms, ethanol, as the product of microbial fermentation, has become one of the oldest forms reflecting the application of microbial biotechnology[1]. Today, the use of ethanol is no longer limited to the beverage; the high octane number of ethanol means that can be mixed with existing fuel sources and is an important part of the solution to the energy crisis and environmental problems[2].

Ethanol fermentation is a biological process performed by *Saccharomyces cerevisiae* (*S. cerevisiae*), one of the first model organisms to be studied scientifically, but the discovery of this process took generations of scientists (Figure 1). Ethanol fermentation was first studied in 1789 by the chemist Antoine Levoisier, who worked out the equation for how sucrose was converted to ethanol: grape must =carbonic acid + alcohol[3]. However, it was not until 1843 that the exact equation '$C_6H_{12}O_6 \rightarrow 2C_2H_5OH + 2CO_2$' was calculated by Dumas. Until then, *S. cerevisiae* was not considered a living organism. It was only through the introduction and use of microscopic equipment that the yeast cell was described by Schwann in 1837[3], and the 'cell theory' that living structures originate from the formation and differentiation of units was subsequently proposed. With the development and maturation of the ethanol fermentation industry, the corresponding research became more in-depth. Between 1855 and 1875, Pasteur responded explicitly to the identity and role of yeast and proposed that fermentation was a physiological phenomenon that could be divided into aerobic and anaerobic forms. In 1857, Pasteur published his first paper on alcoholic fermentation, explaining that the breakdown of sugars into alcohol and carbonic acid was associated with life processes, and that sugars provided nutrients for yeast growth. Three years later, Pasteur reported a figure for the efficiency of ethanol fermentation, stating that only 95% of the sugar was converted to ethanol, while

the remaining 5% was converted to byproducts such as glycerol, succinic acid, and 'cellulose'[4]. In the same year, Berthelot proposed that *S. cerevisiae* used hydrolase to decompose sucrose into monosaccharides. The mechanism of ethanol synthesis in *S. cerevisiae* has been continuously explored, which has promoted people's concern for microbial biotechnology applications. Emphasis has been placed on enhancing the development of *S. cerevisiae* biosynthesis at the genetic manipulation level. It was not until 1996 that the whole genome sequencing of *S. cerevisiae* was completed, which opened the prelude to the molecular level research of *S. cerevisiae*[5].
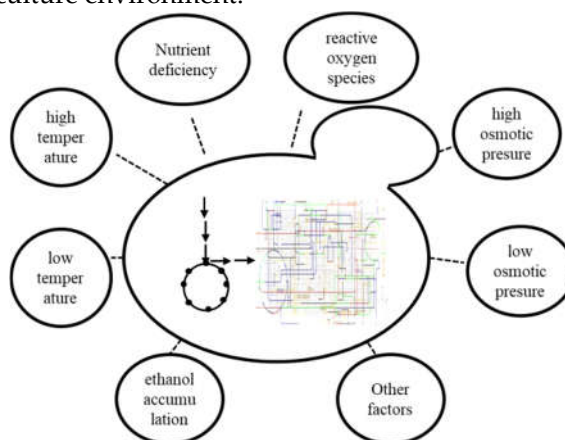

Figure 1. The cognitive history of S. cerevisiae ethanol fermentation.

S. cerevisiae is a well-studied model that has facilitated understanding of eukaryotic processes, and it was the first eukaryotic genome to be fully sequenced[5]. However, *S. cerevisiae* is not uniformly domesticated; strains vary in performance in different regions and have their own adaptability to each environment. With the increasing demand of people for material culture, as well as the demand for environmental protection, means there are increasing requirements on the amount of bioethanol synthesis. Therefore, the production capacity of biosynthetic ethanol will need to be greatly improved. Numerous strategies are proposed to facilitate an increase in bioethanol production, including: 1) using high-quality and cheap substrates such as starch, cellulose, and molasses; 2) improving the fermentation process and reducing energy consumption, such as through ethanol distillation; 3) obtaining robust strains to improve production performance, including speed and quality; and 4) achieving high-density fermentation. The advancement of each of these strategies is a huge boost to ethanol biosynthesis. In the U.S.A., 199 bioethanol synthesis plants have been built (up to 2022, https://www.biofuelsdigest.com/bdigest/) in 25 states, with a total capacity of 53.6 million tons per year, and the U.S. government continues to increase funding for this technology.

Despite the increasing demand and investment, ethanol fermentation still faces multiple challenges at present (Figure 2). Ethanol synthesis by *S. cerevisiae* can be conducted in different regions and different environments, and can be achieved through the degradation of different polysaccharides. However, the efficacy of the wild-type strain itself is often unable to meet the needs of the production design, so it faces a variety of stress factors, as follows. 1) Influence of high temperature, including biological metabolism heat production, mechanical stirring heat production, and regional high temperature climate and other factors[6, 7]. 2) The effect of low temperature. For example, after the strain cryogenic preservation process, performance of the fermentation strain needs to be maintained[8-10]. 3) The effect of nutrient deficiency. Glucose and sucrose are the best carbon sources for fermentation and are used preferentially when other carbon sources are present. However, sudden depletion of glucose and sucrose presents a precipice of carbon metabolism and utilization (After the best carbon source is used, the carbon source that is used later will not be used immediately), and then utilization of other carbon sources commences[11]. 4) Influence of high osmotic pressure. Increasing the substrate concentration has become one of the most direct means to improve the degree of fermented wine and reduce the energy consumption of subsequent distillation[12, 13]. 5) Influence of low osmotic pressure. For that need to go through the washing step in the process of seed solution cultivation, multiple infusions of water will cause cell hypoosmosis and cell swelling[10]. 6) Influence of ethanol accumulation. Studies have shown that most microbial

growth is affected by 2% (v/v) ethanol concentration[6]. 7) The effect of reactive oxygen species (ROS). Various adverse factors in the fermentation process can aggravate the accumulation of ROS in cells, resulting in lipid oxidation of the cell membrane and nucleic acid damage[14, 15]. 8) Other influences. Additional adverse factors in the substrate environment include the high number of ions in molasses and the influence of CO_2 on culture environment.

Figure 2. Multiple challenges in ethanol synthesis of S. cerevisiae.

Improving understanding of *S. cerevisiae* and the potential problems of the fermentation environment will enhance the efficiency of ethanol biosynthesis. The following sections focus on current ethanol synthesis processes using glucose, sucrose, starch, and molasses as substrates, and summarize the factors known to affect production.

2. Ethanol fermentation with saccharomyces cerevisiae

2.1. Ethanol fermentation based on glucose and sucrose

The best carbon source for ethanol fermentation by *S. cerevisiae* is glucose or sucrose. Increasing the concentration of such substrates is one approach to achieve a high concentration of ethanol to reduce subsequent distillation energy. However, with the increase of substrate concentration, the osmotic pressure of the culture environment rises, and hyperosmolality can cause volume shrinkage of cells, which stresses the growth and vitality of cells[12, 13]. In order to overcome the influence of high osmotic pressure and high ethanol concentration feedback inhibition, ethanol recovery with plate membrane pervaporation device[16], extraction fermentation[17], electrostatic fermentation[18], ultrasonic fermentation[19], immobilized fermentation[20], multi-stage reaction tower recycling fermentation[21-23] and so on have been widely studied (Table 1). But when the carbon source is used by *S. cerevisiae* to make ethanol, not only do these problems of high osmotic pressure and ethanol feedback inhibition occur, when multiple carbon sources are present, glucose and sucrose are always consumed first. The presence of these sugars inhibits gluconeogenesis, glyoxylic acid circulation, respiration (known as the 'Crabtree effect'), and the intake of fewer carbohydrates. This pathway inhibition by glucose ensures that preferred sugars are metabolized before consumption of replacement carbohydrates such as maltose and galactose. However, this process can have two serious consequences. 1) Prolonged use of glucose and sucrose can result in yeast cells reducing their ability to metabolize other sugars. 2) The catabolic inhibition caused by glucose does not begin immediately with the depletion of glucose; instead, there is a lag period that causes a marked drop in carbon metabolism and affects growth[11].

Table 1. Ethanol fermentation based on different substrates.

Substrate	Method	Achievement	Ref
Glucose	Coupled to a flat-plate membrane pervaporation unit to recover ethanol	ethanol productivities reached 7.8 g/L/h	[16]
Glucose	Extractive fermentation, aqueous feeds with 413 and 495 g/L glucose	Ethanol conversion efficiencies reached 90%-95% conversion	[17]
Glucose	electrostatic fermentation with 15 V	The consumption of glucose reached 98% in 20 h	[18]
Glucose	Fermentation with mild ultrasonication	10 times faster than non-stirred fermentation.	[19]
sucrose	Continuous potable production by immobilized on mineral kissiris	ethanol productivities reached 10 g/L/h	[20]
sucrose	Fermentation with self-flocculating yeast in a tower upflow reactor	ethanol productivities reached 13.5 g/L/h	[21]
sucrose	Continuous ethanol fermentation in tower reactors with cell recycling	ethanol productivities reached 18 g/L/h	[22]
sucrose	Addition of air at a rate of 150 dm^3 /min/m3 of reactor volume during the first 12 h	35% sucrose is consumed and 16% ethanol is fermented	[23]
Starch	Co-cultured with amylolytic yeast	fermented unhydrolyzed starch to ethanol with conversion efficiencies over 90% of the theoretical maximum.	[24]
Starch	Starch Pretreated with 0.5 M HCl at 60℃, followed by combined actions of a-amylase and glucoamylase.	Ethanol conversion efficiencies reached 95%	[25]
Starch	a raw starch hydrolyzing enzyme was used that converts starch into dextrins at low temperatures, and hydrolyzes dextrins into sugars	Ethanol conversion efficiencies reached 88.4%	[26]
molasses	Batch and semicontinuous immobilized yeast fermentations together with a pervaporation method	Residual sugar was reduced 42%, ethanol produced increased 18.1%	[27]
molasses	batch and continuous fermentations of blackstrap molasses using immobilized yeast cells on thin-shell silk cocoons	Ethanol concentration 11.5% higher than produced by free cells	[28]
molasses	A new candidate ethanol fermentation-related regulatory gene, PHO4, was replaced from a low ethanol yield but rapid growth strain to a high ethanol yield industrial strain.	Average high ethanol production reached 114.71 g/ L, ethanol production increased by 5.30% The fermentation time was 12.5% lower than that of the original strain.	[29]

2.2. Ethanol fermentation based on starch

Starch is a cheap and renewable carbon source[30] composed of a linear chain of α-1, 4-linked D-glucopyranose units (amylose) and the highly branched amylopectin fraction that consists of α-1,4- and α-1,6-linked D-glucopyranose units. *S. cerevisiae* cannot degrade starch naturally. Starch has to be hydrolyzed through liquefaction and saccharification before use, and efficient starch hydrolysis requires both an α-amylase and a glucoamylase together with an α-1,6-debranching activity[31]. α-Amylase randomly cleaves the α-1,4-glycosidic linkages inside the starch granule and degrades the whole structure to release non-reducing ends. Subsequently, glucoamylase cleaves α-1,4- and α-1,6-glycosidic linkages from the non-reducing ends. Considering that oligosaccharides produced during decomposition can inhibit α-amylase, it is necessary to adjust the ratio of both enzymes during use; the addition of pullulanases (which hydrolyze the α-1,6-glycosidic bonds) can also greatly improve the efficiency of starch hydrolysis[32]. Industrial production of sugars from starch usually consists of four steps—jet-cooking, addition of α-amylase, cooling (regulating pH), and addition of

glucoamylase—to yield sugar streams that can be used for ethanol fermentation[33]. The boiling step can consume a lot of energy—approximately 10–20% of the bioethanol price, with further damage caused by the Maillard reaction. Consequently, some factories have changed the 'boiling process' to a 'cold process' by adding raw starch-degrading enzymes, saving approximately 40–50% of the total cost[34, 35]. In addition, pretreatment of starch[25] or co-culture with bacteria with starch decomposition ability[24] has also become a feasible research content (Table 1).

The demand for energy is increasing worldwide—global energy consumption is predicted to rise by more than 30% between 2018 and 2050, while bioenergy would increase by 3% per year during that time[36]. This increasing energy demand and the emergence of the global food crisis is driving the use of high-starch plant substitutes for ethanol production. Cassava (72.6–76.6% starch for dried cassava) is a very attractive raw material for bioethanol production that is not affected by food and feed shortage concerns[37]. A repeated batch fermentation of cassava yielded an ethanol concentration of more than 83.64 g/L[38]. The small aquatic plant duckweed (35.7% amylose and 64.3% amylopectin[39]) is another promising source for bioethanol production. Duckweed could be hydrolyzed with α-amylase and amyloglucosidase (2:1, by vol.), with the final ethanol concentration reaching 0.19 g ethanol/g dry biomass[36].

Starch needs to be hydrolyzed before it can be used by *S. cerevisiae*, and although progress has been made to make the process more efficient, adding enzymes and other reagents ultimately increases the cost of the process. Therefore, development of a recombinant *S. cerevisiae* that co-expresses the required glucoamylase and a-amylase has been explored. Many genes from filamentous fungi and yeasts that encode amylolytic enzymes have been expressed in *S. cerevisiae*[40-42]; However, only a limited number of fungal and bacterial strains meet the standard for commercial amylase production. A major challenge is in the simultaneous production of these amylolytic enzymes that exhibit high substrate affinity and specific enzyme activity[43]. A recombinant *S. cerevisiae* combined with exogenous enzymes to promote starch hydrolysis for ethanol fermentation has been engineered and its carbon source conversion rate can reach 94%[44]. The rapid development of synthetic biology may direct further developments of recombinant strains of *S. cerevisiae* for ethanol fermentation using starch, but this also needs to be approved for use in the corresponding part (Food regulatory agency et al).

2.3. Ethanol fermentation based on molasses

No matter how volatile the world is, humans cannot live without food and energy. Consequently, the development of non-food resources for energy supply is needed for robust and sustainable global energy, and strengthening the use of molasses is an important component of this process. Molasses is a by-product of the sugar industry (3 tons of sugar produces approximately 1 ton of molasses) that is generated during sugarcane and sugar beet processing. It contains 30–60% (w/v) sugar, plus colloids and a large number of metal ions[27, 45, 46].

Molasses generally requires a pretreatment process before it can be used directly by *S. cerevisiae*. This pretreatment usually involves boiling, adding concentrated sulfuric acid to adjust the pH, cooling overnight, reheating and adding activated carbon, centrifugation, and adding hydrated lime. Despite the short pretreatment process, according to Professor Abbott's 'alert level' theory[47], there are still many factors in molasses that can stress the growth and vitality of cells. To address this issue, scholars have used semi-continuous (feed) fermentation [48, 49], ceramic microfiltration[27], adsorption column[50] or dialysis ultrafiltration[51] to reduce the influence of multiple stresses during molasses fermentation. In addition, immobilization methods for yeast, such as calcium alginate[52], bagasse[53], and thin-shell silk cocoon[28](Table1), have been employed to increase the activity of yeast cells. The alcohol content in the mash made by yeast cells using molasses was improved to a certain extent by these modifications. However, the inherent genetic properties of yeast cells mean the growth and vitality of yeast cells are still limited under various stress pressures. Therefore, the research of gene editing for genetic properties to improve production has become a development, such as ethanol regulating gene replacement[29] and so on. Now, ethanol fermentation with high concentrations of molasses remains a challenge.

To overcome the genetic limitations of *S. cerevisiae*, the resistant strains of *S. cerevisiae* based on the sucrose addition model have been studied and the tolerance of these yeast cells to ethanol, osmotic pressure, and oxidative stress has been reported[54, 55]. However, the research on ethanol fermentation with high concentration molasses has had little effect. A careful taste reveals that ethanol fermentation using molasses has a lower degree of fermented liquor in the limos compared with the fermentation using sucrose, which means that ethanol feedback inhibition is not the main factor affecting ethanol fermentation from molasses. Similarly, the osmotic pressure formed at high sucrose concentration did not cause lethal effects on *S. cerevisiae*. Therefore, identifying why *S. cerevisiae* struggles to use high concentration molasses for ethanol fermentation is crucial for future research. Our unpublished research indicates that the influence of K^+ and Ca^{2+} in molasses may be a major cause of this difficulty (Date will be published in another paper). However, there is a current lack of detailed reports on the influence of ions on molasses in specific environments. Therefore, it will be an effective method to reveal the stress factors with robust strains of *S. cerevisiae* cultured under the environment of molasses fermentation for specific industrial production and combined with multi-omics analysis.

3. Conclusions

S. cerevisiae ethanol fermentation has made a great contribution to the development of bioenergy by humans. *S. cerevisiae* is a living, intelligent organism, and if we want to direct its efficient biosynthesis with human will, we must understand *S. cerevisiae* itself and its current production predicaments. Glucose and sucrose are the best carbon sources for *S. cerevisiae*. There is potential for ethanol synthesis based on starch and molasses as substrates, as well as fermentation based on cellulose. However, these substrates impose various restrictive factors on the growth and metabolism of S. cerevisiae Domestication of wild-type strains of *S. cerevisiae* takes a long time and there are many uncertain factors. However, the development of synthetic biology is anticipated to facilitate the directional construction of a new generation of engineered *S. cerevisiae* that may balance the current situation of ethanol production. The caveat is that identification of the accurate target site and target direction is needed to achieve this approach.

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