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REVIEW ARTICLE

CURRENT APPROACHES TO EFFICIENT BIOTECHNOLOGICAL PRODUCTION OF ETHANOL

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Abstract
Current researches on ethanol production and development deals with process engineering to improve bioprocessing steps. In this paper the key role of effective technologies is recognized through the analysis of major trends in industrial biotechnological process and optimization related to ethanol production. Yeast strains of *Saccharomyces cerevisiae* have been used in baking, brewing, and wine-making industries, and were extensively studied in recent years for ethanol production, in which yeast cells are subject to improving of culture conditions, including nitrogen limitation, high temperature, ethanol inhibition, the osmotic stress from substrate sugars, and so on. Industrial yeasts must sense and response to the stress conditions rapidly and adapt to these adverse environmental factors by adjusting their metabolic activities to avoid substantial viability loss. The capability of cells to tolerate various stresses is one of the important criteria to select industrial strains for efficient ethanol fermentation.
Finally, some concluding considerations on current and future research tendencies in ethanol production are presented.

Key words: yeast starter cultures, industrial fermentation, ethanol production.

Introduction

Ethanol production is among the oldest technology and is produced commercially by fermentation of cereal grains, molasses or other materials with high starch and/or sugar contents. The fermentation process involves conversion of sugars to alcohol and carbon dioxide by the yeast *Saccharomyces cerevisiae.*

The principal biological agents of fermentation are yeasts belonging to the genus *Saccharomyces*, which can catalyse alcoholic fermentation to ethanol production. In recent years, yeast strains of *Saccharomyces cerevisiae* were extensively studied for biotechnological properties enhanced. Industrial yeast strains have to resist to the stress conditions rapidly and to adapt easy by modifying their metabolic activities to avoid substantial viability loss.

The capability to tolerate various stresses is one of the important criteria to select the most effective industrial strains for efficient ethanol fermentation.

Recent advances in genomics and bioinformatics have made it possible to elucidate when and why yeasts produce ethanol in high concentrations, and how this remarkable trait originated and developed during their evolutionary history.

Besides the common stress conditions such as ethanol toxicity and osmotic pressure, yeast strains for ethanol production are also expected to tolerate high temperature since ethanol fermentation under relatively low temperature cannot be operated in summer (or when the environmental temperature is high) with the regular cooling water.

Ethanol is produced through the fermentation of agricultural by-products such as sugarcane, corn and wheat, sugar beet and cassava, among others.

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The great majority of ethanol produced in the world is from sugarcane molasses.

The objective of this paper is to review the state of the art in industrial bioethanol production from a bioprocess engineering point of view for productivity improvement.

Microorganisms used for industrial bioethanol production

During the time many microorganisms have been studied for ethanol production, but *Saccharomyces cerevisiae* still remains as the first species. Other yeast as *Schizosaccharomyces pombe* presents the advantage of tolerating high osmotic pressures (high amounts of fermentescible sugar) and high solids content. However, a fermentation process using a wild strain of this yeast has been patented (Carrascosa, 2006). Bacterial *Zymomonas* species has also been intensively studied over the past three decades and repeatedly claimed by some researchers as an alternative to *Saccharomyces cerevisiae* utilisation in ethanol production.

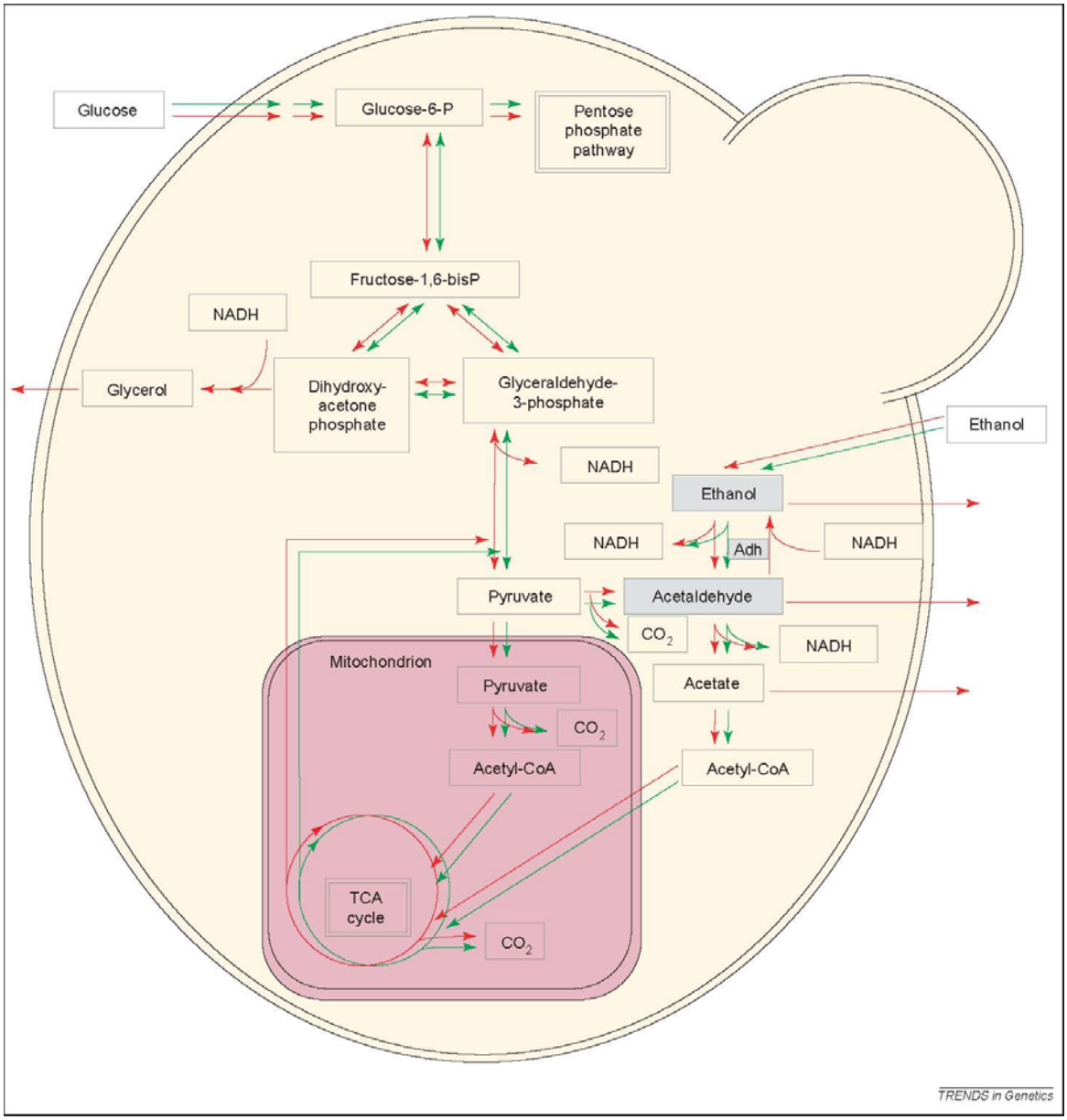

Figure 1. *A scheme of the pathways involved in glucose and ethanol assimilation under aerobic conditions underlining the differences between a Crabtree-positive yeast, Saccharomyces cerevisiae (red arrows), which can accumulate ethanol, and a Crabtree-negative yeast Kluyveromyces lactis (green arrows), which degrades hexoses directly to CO_2 The conversion between acetaldehyde and ethanol, catalyzed by alcohol dehydrogenase. Abbreviation: TCA cycle, tri carboxylic acid cycle (Piskur et al., 2006)*

Many researchers studied the ethanol fermentation with *Saccharomyces cerevisiae* strains but in some cases a lack of recognition of its metabolic pathway led to approaches that are unlikely to yield significant improvements. The fundamental physiological characteristic of ethanol producing yeasts is their ability to degrade carbohydrates, six-carbon molecules (C6) - glucose, to two-carbon components (C2) - ethanol, without completely oxidizing them to CO_2, even in the presence of oxygen, as many other yeast do. Yeasts such as *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*, which accumulate ethanol even in the presence of oxygen are called Crabtree-positive yeasts, whereas those that degrade sugars to CO_2, such as *Kluyveromyces lactis* are designated as Crabtree-negative yeasts (Pronk *et al.*, 1996). During ethanol production, the energy for growth is provided by the glycolysis and fermentation pathways rather than by the oxidative respiration pathway (Fig. 1).

Beside to ethanol and CO_2 formation, different by-products are also produced during ethanol fermentation such us glycerol, organic acids and higher alcohols. But the glycerol is the main one. Higher pH, increased osmotic pressure, lower flux of pyruvate due to the utilization of glycolytic intermediates subsequent to the step in the pathway producing reduced NAD for biosynthesis all can stimulate the conversion of dihydroxyacetone phosphate to glycerol (Ingledew, 1999).

Other by-products are organic acids and higher alcohols, but they are produced at much lower levels. The formation of these by-products as well as the growth of yeast cells inevitably direct some glycolytic intermediates to the corresponding metabolic pathways, decreasing the ethanol yield to some extent.

In the industry, the ethanol yield is calculated based on the total sugar feeding into the fermentation system without deduction of the residual sugar and can be as high as 90-93% of its theoretical value of ethanol bioconversion to glucose (Ingledew, 1999). The residual sugar can be controlled at a very low level. Any ethanol fermentation research which is expected to be practical needs to bear these criteria.

Yeast cell stress

During ethanol fermentations, yeast cells are negative affected from various stress factors. Some are environmental such as nutrient deficiency, high temperature and contamination, while the others are from the yeast cell metabolism such as sugar high content tolerance, ethanol tolerance and production and its corresponding inhibition on yeast cell growth and ethanol production, especially under very high gravity conditions. Some of these stress factors are depicted in Fig. 2.

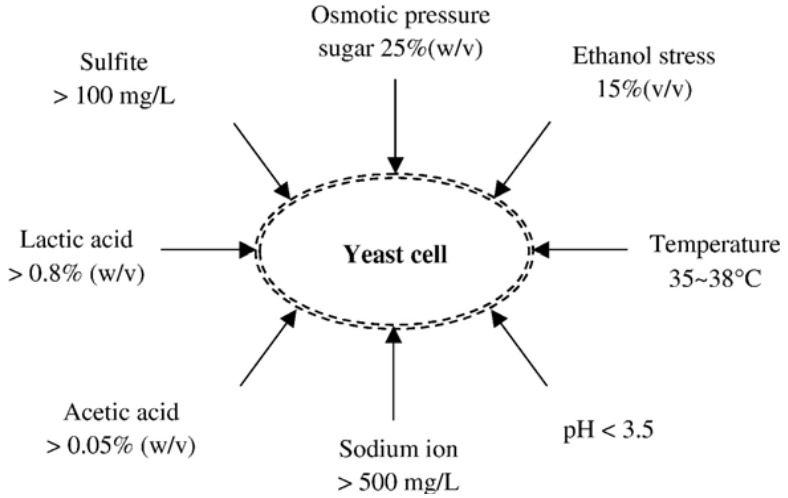

Figure 2. *Potential environmental stresses on Saccharomyces cerevisiae during alcoholic fermentation (Ingledew, 1999 cited by Bai et al. 2008)*

Many of them are synergistic, affecting yeast cells more severely than any single one, leading to reduced yeast viability as well as lower ethanol yield.

Stress can cause structural changes and metabolic changes in an organism acting as expression activator for genes involved in the synthesis of specific compounds that protect cells (Banat *et al*., 1998).

The factors triggering the expression of this type of genes can be biotic or abiotic. Biotic factors induce changes in the gene expression of the guest, giving rise to the synthesis of specific compounds that generate resistance to the strange organism. Abiotic factors can be temperature, osmotic stress, anaerobic conditions, heavy metals, growth regulators, ultraviolet radiation, metabolic repressors, and pH (Rajoka *et al*., 2005, Cakar *et al*., 2005).

Although many problems still exist among the studies of the mechanisms of ethanol inhibition, it has been accepted that the membranes of some organelles and cells are the main targets of ethanol attack (D'Amore and Stewart, 1987).

Ethanol inhibition has multiple effects and is very complicated. Some possible sites in yeast cells at which ethanol could exert a significant influence are shown in Fig. 3.

The inhibition of ethanol is favoured by the presence of other fermentation by-products such as acetaldehyde and acetate, and other stresses such as high temperature (Bai *et al*., 2008).

It was demonstrated that especially unsaturated fatty acids such as palmitoleic acid (C16:1) and oleic acid (C18:1), are key membrane components, which counteract ethanol inhibition by increasing the fluidity of the plasma membranes, in compensation for fluidity decreases resulting from ethanol effects.

The levels of these two unsaturated fatty acids were measured to be higher for ethanol tolerant strains, or significantly increased after ethanol stress was exerted (You *et al.,* 2003). They are synthesized in *Saccharomyces cerevisiae* by the catalysis of the oxygen and NADH-dependent desaturase of palmitic acid (C16:0) and stearic acid (C18:0).

Therefore, a small amount of oxygen is required for yeast cells to synthesize these unsaturated fatty acids under anaerobic fermentation conditions. The role of a small amount of oxygen supply in improving the ethanol tolerance of yeast cells was investigated as early as in the 1980s (Ryu *et al*., 1984) and has been practiced in the ethanol fermentation industry for a long time. It is predicted that, under a very high gravity fermentation condition, the role of such "micro-aeration" in improving ethanol tolerance will be more significant since much stronger ethanol inhibition can develop.

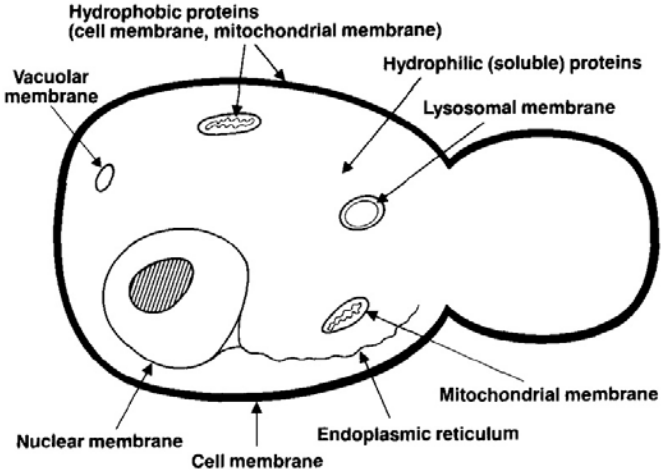

Figure 3. *Possible target sites for ethanol inhibition in yeast cells (D'Amore and Stewart, 1987 cited by Zhao and Bai, 2008)*

Importance of amino acid biosynthesis and transport for ethanol tolerance and cell viability was also reported in later studies (Hirasawa *et al*., 2007; Pham and Wright, 2008). The amino acid supplementation with leucine, alanine, tyrosine, phenylalaine and methionine was proposed to

contribute to improved stress tolerance and ethanol yield.

The presence of ethanol does not only inhibit cell growth, but also represses glucose transport, thereby leads to defect in yeast. Therefore the tolerance of sugar transporters to ethanol inhibition is an important feature to consider when selecting strains for efficient ethanol production (Santos *et al.*, 2008). It is very important to choose yeast cells before the total bioconversion of residual sugars to ensure good cell viability and ethanol fermentation performance for cell reutilization.

Stress due to temperature has been the most studied abiotic factor, where both heat and cold induce the synthesis or storing of a group of proteins that increase stress resistance.

Some successful attempts to adapt yeasts to high temperatures have been described. *Saccharomyces cerevisiae* yeasts, capable of fermenting at 40 and 45°C, have been obtained using progressive cultures (Rikhvanov *et al.*, 2001). Thermotolerant yeast strais have been obtained by selecting survivors after a shock process at relatively high temperatures. Some authors demonstrated that during 15 min of incubation at 55 °C resulted tolerant cells to higher temperature than non-incubated controls.

Morimura *et al.* (1997) developed by protoplast fusion and manipulating culture conditions, flocculating strains capable of growing at 35 °C and at molasses concentration of 22% (w/v). Under these conditions and using repeated-batch cultures at laboratory scale, ethanol concentration of 91 g/L and productivities of 2.7 g/(L·h) were obtained.

To avoid the negative influence of stress factors is through the conditioning of molasses by the addition of different compounds capable to neutralize the inhibitory effects of the medium components. Fermentescible substrates (molasses) should be supplemented with nutritional factors promoting the yeast growth like EDTA, ferrocyanide and zeolites (Castellar *et al.*, 1998), commercial enzymatic complex of amylases, cellulases and amylopectinases which allows the conversion of non-fermentable substances into assimilable compounds improving the alcoholic fermentation (Acevedo *et al.*, 2003). On the other hand, the addition of a minimum inhibitory concentration of acids to molasses will stop bacteria growth, increase ethanol yields and avoid the need for antibiotics as described in the patent elaborate of Maye (2006).

Fermentation substrates

The various substrates used for ethanol production and their processing are briefly summarised below.

1. Sugar crops, e.g., sugarcane, sugar beet, sorghum, etc. provide a good substrate. Juices from these crops contain simple fermentecible sugars, e.g., sugarcane juice has about 12% sugars, and can be used directly for fermentation.

2. Bye product sugars from crop processing, e.g., molasses, sweet sorghum syrup, and spent sulphite liquor are the most common substrates. Molasses obtained after sugar recovery contains about 50-55% total fermentecible sugars. Molasses is first suitably diluted before being used for fermentation (Fig. 4).

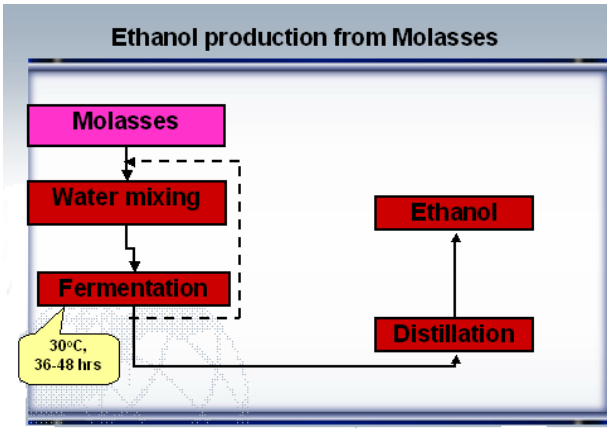

Figure 4. *Ethanol production from molasses*

3. Cereals like maize, wheat, sorghum, etc. contain 60-75% w/w starch, which on hydrolysis produces glucose in the ratio 9: 10. Generally, starch is a mixture of amylose (20-30%; water soluble linear polymer) and amylopectin (70-80%; water insoluble branched polymer).

Conversion of starch into glucose is usually achieved by a thermal process aided by enzymes (Fig. 5). Cereals are milled (wet or dry) and the resulting powder is mixed with water in the ratio of 1:2.5-3.

Enzyme α-Amylase is added and the temperature is raised by steam injection to 105-110°C and held for about 20 min. α -Amylase can be obtained from thermoresistant bacteria like *Bacillus licheniformis* or from engineered strains of *Escherichia coli* or *Bacillus subtilise*. α -Amylase is used during the first step of hydrolysis of starch suspensions. During this period, starch grains become dissolved to form a viscous suspension (gelatinisation) due to the thermal effect of heat. At the same time α-amylase hydrolyses partially some of the starch molecules; this reduces the viscosity of the suspension, process which is called liquefaction. The product of this first step is a starch solution containing dextrines and small amounts of glucose.

In last years, the possibility of hydrolysing starch at low temperatures for achieving energy savings is being investigated (Robertson *et al.*, 2006).

The suspension called mash is cooled to 85-90°C, and new quantity of α - amylase is added to complete the process of liquefaction (for 90 min). The total dose of α-amylase is about 1.5 kg/ton starch. The mash is cooled to 55-60°C, and 1.5-3.5 kg glucoamylase/ton of starch is added to produce glucose from dextrins and maltose. The glucoamylase is obtained generally from *Aspergillus niger* or *Rhizopus sp.* (Shigechi *et al.*, 2004). The reaction duration is usually 1-2 days, and it achieves the dextrose equivalent of 99. Saccharification by glucoamylase continues during the fermentation.

Fermentation is performed using *Saccharomyces cerevisiae* and is carried out at 30-32 ^{o}C with the addition of ammonium sulfate or urea as nitrogen sources. In the case of corn mashes proteases can be added to the mash to provide an additional nitrogen source for the yeast resulting from the hydrolysis of corn proteins (Bothast and Schlicher, 2005).

Fermentation of wheat mashes of very high gravity (VHG) has been proposed as well. These mashes consist of wheat starch hydrolyzates containing 300 g or more of dissolved solids per liter of mash. VHG fermentation technology implies that high ethanol concentrations are obtained from very concentrated sugar solutions (Thomas *et al.*, 1996).

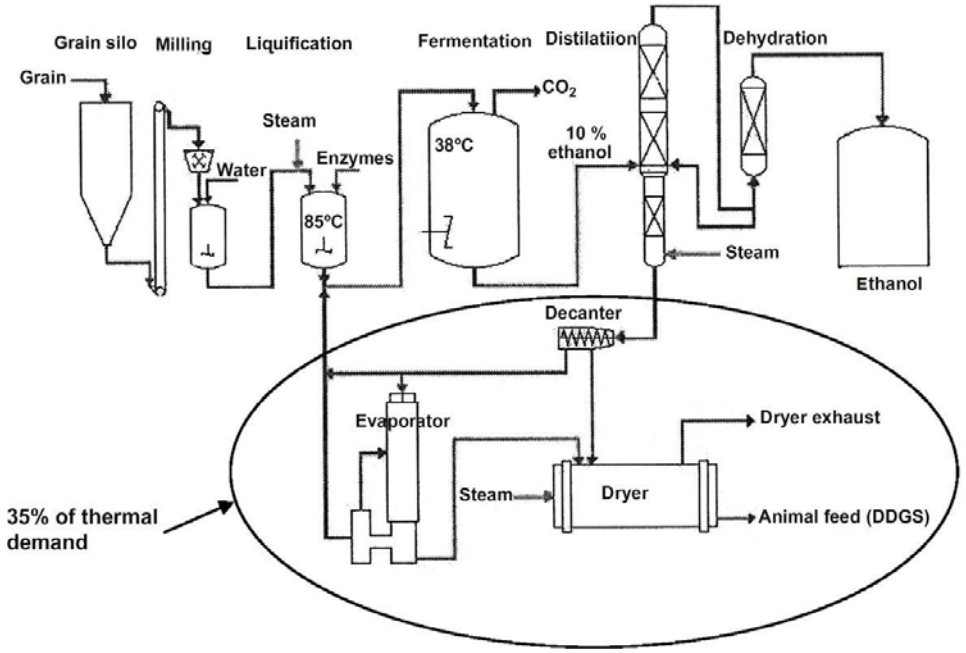

Figure 5. *Process of generating ethanol from wheat (Murphy and Power, 2008)*

4. Tubers like cassava, yams, potato etc. are rich in starch (30% on fresh weight basis). Cassava tubers are washed, mashed to pulp and subjected to liquefaction and saccharification in a manner similar to cereals.

Cassava tuber roots can be air dried to 15% moisture and stored; tuber chips or meals are better for storage (Fig. 6).

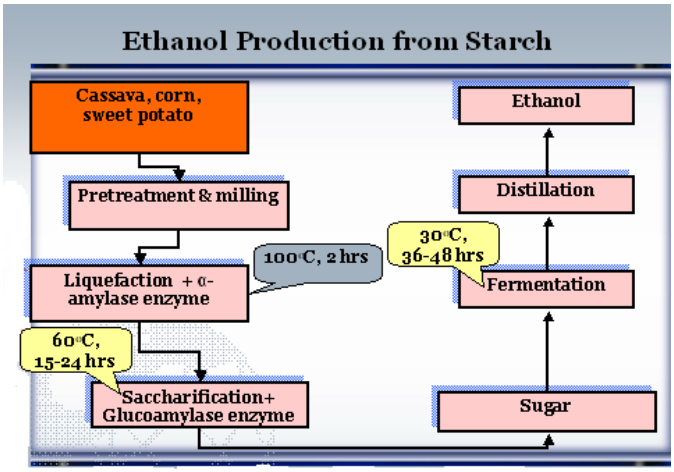

***Figure 6**. Ethanol production from starch raw material sources*

Ethanol production from cassava can be accomplished using either the whole cassava tuber or the starch extracted from it. Starch extraction can be carried out through a high-yield large-volume industrialized process as the Alfa Laval extraction method or by a traditional process for small- and mid-scale plants. This process can be considered as the equivalent of the wet-milling process for ethanol production from corn. The production of cassava with high starch content (85–90% dry matter) and less protein and minerals content is relatively simple. Cassava starch has a lower gelatinization temperature and offers a higher solubility for amylases in comparison to corn starch. The hydrolysis of cassava flour has been proposed for the production of glucose in an enzymatic hollow-fiber reactor with 97.3% conversion (Lopez-Ulibarri and Hall, 1997) considering that cassava flour production is more simple and economic than cassava starch production. It is considered that cassava ethanol would have better economic indicators if the whole tuber is used as feedstock, especially when small producers are involved (Sanchez and Cardona, 2008).

5. Cellulosic substrates are the most abundant.

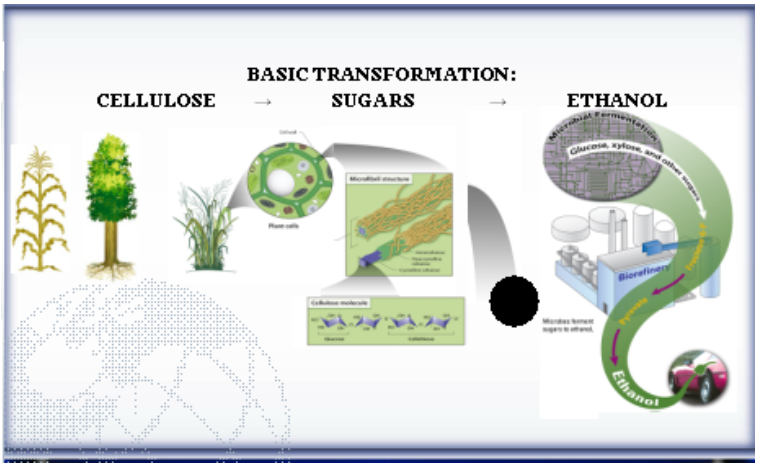

***Figure 7**. Basic transformation of cellulose to ethanol*

Acid hydrolysis of cellulose is technically possible and was very used (Fig. 7).

Enzymatic hydrolysis of the substrate has presented problems.

A more promising approach appears to be the direct conversion of suitably pretreated cellulose into ethanol by mixed cultures of celluloytic and fermenting bacteria.

Some thermophilic *Clostridium* species are rather promising. The pretreatment of cellulosic biomass may be either physical or chemical and is aimed at modification of the substrate to increase the cellulose hydrolysis potential (Fig. 8).

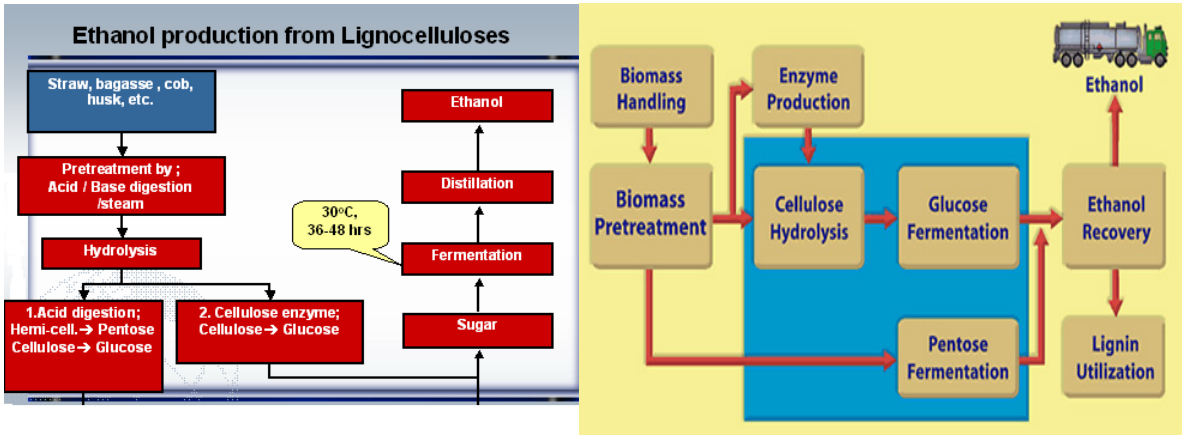

Figure 8. *Ethanol production from lignocelluloses*

Fermentation processes

Typically, fermentation of 100 g glucose by selected strains of *Saccharomyces cerevisiae* yields 45-49 g ethanol, the theoretical limit being 51.1 g ($C_6H_{12}O_6$ --> $2C_2H_5OH + 2CO_2$). Both batch, semi continuous and continuous fermentation processes are used, and often yeast cells are recycled to increase the economical efficiency.

The Melle-Boinot process is the typical process for ethanol production by batch fermentation. This process comprises the weight and sterilization of feedstock, followed by the adjustment of pH with H_2SO_4 and of the degrees Brix to values of 14-22. Obtained media is fermented by yeasts. The fermentative product is decanted, centrifuged and sent to ethanol separation stage, whereas the yeast biomass are recycled to the fermentation in order to reach high cell concentration during cultivation (Kosaric and Velikonja, 1995).

Fed-batch culture implies controlled levels of substrate concentration during the course of fermentation, while ethanol is accumulating in the medium.

By using multiple or repeated batch fermentation, the use of flocculating yeast strains plays an important role. In this process, after starting a conventional batch, the yeasts are decanted in the same vessel where they were cultivated by removing the clarified culture broth.

Then, an equal amount of fresh culture medium is added for the following batch. In this way, high cell concentrations are reached and inhibition effect by ethanol is reduced without the need of adding flocculation aids or using separation or recirculation devices.

These repeated batches can be carried out until the moment when the metabolic activity and viability of culture is lost as a consequence of an inhibition of cells metabolism to the fermentation environment factors (Sanchez and Cardona, 2008).

Continuous process uses 3-5 closed vessels. Ethanol concentration increases from 4% in the first vessel to 10% in the final vessel, the productivity ranging from 10 to 20 kg ethanol/m^3hr. In contrast, batch process productivity ranges between 1.5-3 kg ethanol/m^3/hr.

However, final yields are much higher in the batch process, which is the most commonly used.

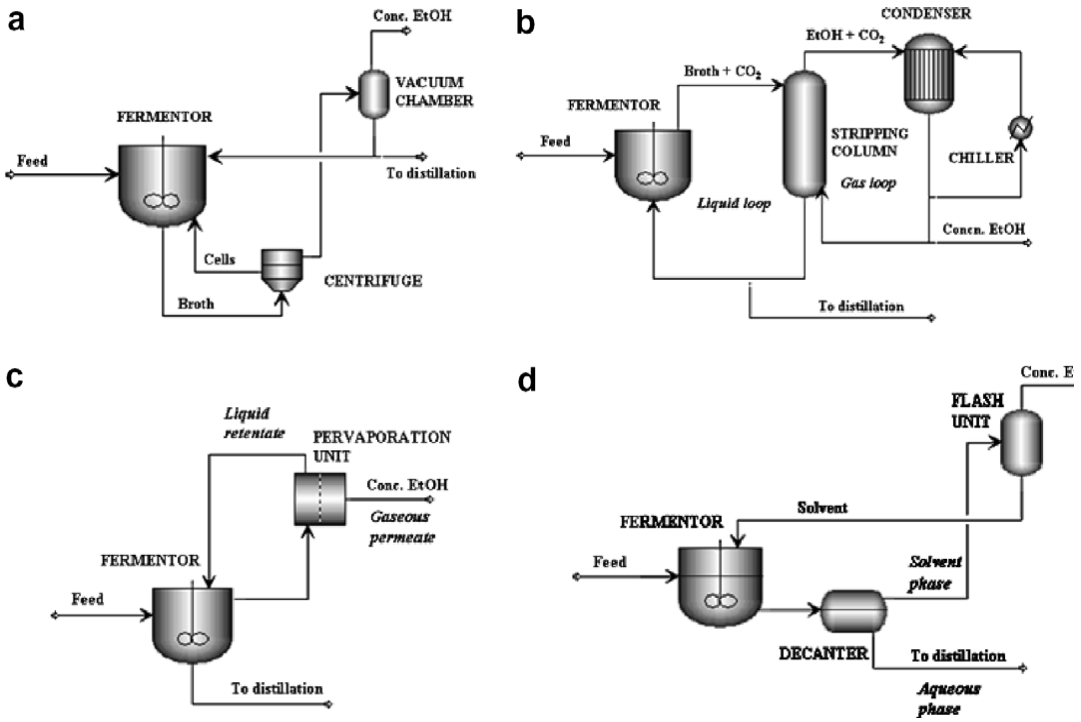

***Figure 9**. Simplified diagrams corresponding to some configurations for ethanol recoverrom culture broth. (a) Vacuum separation, fermentation with recycling of biomass. (b) Fermentation coupled with gas stripping. (c) Fermentation coupled with pervaporation. (d) Extractive fermentation (Cardona and Sánchez, 2007)*

As N source, ammonium sulphate or urea can be used. A salt of phosphoric acid is added to the substrate as P source. Most commonly the temperature during fermentation is 32-38°C and pH is between 4.5 - 5.0. In large fermenter vessels, some cooling system is necessary.

In general, yeast growth stops at 6-9% w/v ethanol, but ethanol production, at least in some strains, may continue up to 15% ethanol or higher.

Ethanol inhibition effect can be relieved by continuous removal of ethanol (Fig. 9).

One of the commercial process, called Biostill technique, passes cell free broth through an evaporation chamber for ethanol removal and this medium is then fed back into the fermenter.

Ethanol recovery is based on distillation. The fermentative broth is distilled in a column to yield 85% v/v ethanol.

The next step of rectification gives 96.5% ethanol, which is then dehydrated to 99.4% using benzene or cyclohexane if the ethanol is to be used as a fuel blend.

Conclusions

Some recommendations should be followed to advance ethanol fermentation technologies with immediate practical applications.

The ethanol fermentation industry is using heterogeneous raw materials rather than pure glucose. The residual sugar at the end of the fermentations is strictly controlled at a very low level, such that the ethanol yield that is calculated based on the total sugar bioconversion into the fermentation systems without deduction of the residual can be as high as 90-93% of its theoretical value of ethanol to glucose.

The ideal yeast strains for ethanol production would be able to tolerate various stress factors. Several successful strategies were applied for the improvement of yeast stress tolerance which resulted in the improvement of ethanol fermentation efficiency.

The high glucose substrate containing 200 g/L total sugars is already used to achieve a corresponding ethanol concentration of 10 (v/v). Low gravity conditions during fermentation significantly increases the energy consumption in the

downstream processes such as the distillation and waste distillate treatment. The very high glucose substrate containing over 250 g/L total sugars is encouraged for further research into ethanol fermentations.

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