

Wine is light,  
held together by water

*Galileo Galilei*

## Chapter 16

### Pulque, wine, and biofuel

Nearly 170 years ago, a short article appeared in *Liebigs Annalen der Pharmacie*, in which chemists made fun of the notion that sugar is converted into alcohol by living organisms. The chemists described little animals that would swallow the sugar, digest it in their stomachs, and secrete alcohol through their intestines and CO<sub>2</sub> through their bladders. They claimed that the bladders of these animals looked like champagne bottles. Chemists at that time believed that the formation of alcohol from sugar was a solely chemical process. The equation for this process had already been set up by Joseph Gay-Lussac (1778–1850) in 1815:

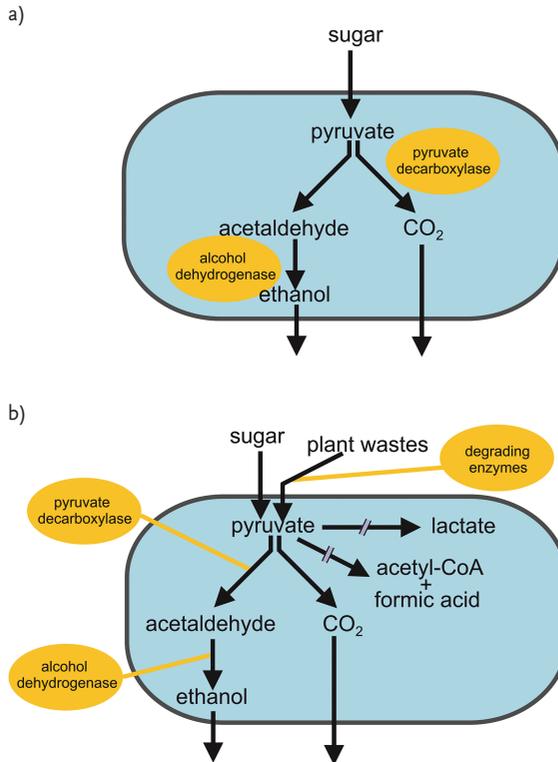


The fact that ethanol fermentation is a biological process was first generally accepted towards the end of the nineteenth century, largely due to the work of Theodor Schwann (1810–1882) and Louis Pasteur (1822–1895), among others.

#### Which organisms are of primary interest as ethanol producers?

First of all, we need to mention yeast, especially the various breeds of *Saccharomyces cerevisiae*, also known as baker's yeast. The yeasts are aerobes as long as oxygen is present, so they respire as we do. However, they grow rapidly in solutions containing around 10 percent sugar, which leads to rapid consumption of the dissolved oxygen. As soon as oxygen is depleted in the fermentation broth, a metabolic switch is activated and glucose is degraded to ethanol and CO<sub>2</sub>. The pathway by which ethanol is produced is rather simple. Glucose with its six carbon atoms is cleaved into two C<sub>3</sub>-compounds. Eventually, two molecules of pyruvate (salt of pyruvic acid) are formed; they each lose CO<sub>2</sub>, and the two compounds formed are reduced to two molecules of ethanol (Figure 32a). The reader should take note of the names of two enzymes involved: pyruvate decarboxylase, which catalyzes the formation of the CO<sub>2</sub> molecules, and alcohol dehydrogenase, which finally produces ethanol.

Now we come to the ethanol-producing bacteria. No doubt, yeasts are the champions when it comes to ethanol production from sugars. We will discuss their applications later. The discovery of bacteria able to produce ethanol by fermentation probably began with Paul Lindner (1861–1945), who was director of the



**Figure 32** Biofuel. (a) Fermentation of sugar to ethanol and  $\text{CO}_2$  as carried out by yeasts and by *Zymomonas mobilis*. (b) Fermentation of sugar and plant wastes to ethanol and  $\text{CO}_2$  by a genetically modified strain of *E. coli*. It is indicated that the genes encoding pyruvate decarboxylase and alcohol dehydrogenase were genetically introduced into the production strain, likewise the genes for degradation of carbohydrates. These genes are expressed

in the cells, and the enzymes thus produced are exported as exoenzymes. Outside the cells, these enzymes degrade plant waste to low-molecular-weight compounds, for example, simple sugars, which can then be taken up by the cells. Furthermore, it is indicated that some metabolic routes are genetically blocked so that primarily ethanol and  $\text{CO}_2$  are produced. (Diagram: Petra Ehrenreich, Goettingen, Germany.)

Institute for Fermentation Industries in Berlin (Germany). From 1924 to 1925, he lived in Mexico as a *consultor tecnico*. There he became familiar with the alcoholic beverage “pulque” brewed by Mexican farmers. They collected agave juice, which undergoes a rapid fermentation and produces within 24 hours a stimulating and pleasant beverage. Paul Lindner discovered under the microscope a “happily motile fermentation bacterium,” which he called *Thermobacterium mobile*. Later it was renamed *Pseudomonas lindneri*, but now it’s called *Zymomonas mobilis*.

This microorganism has specialized in the formation of ethanol from sugars. It is unable to respire with glucose and oxygen as yeast does because it is more or less restricted to carrying out fermentations in the absence of oxygen. The enzymes

mentioned above, pyruvate decarboxylase and alcohol dehydrogenase, are both present in *Z. mobilis*. As a production organism for ethanol on a large industrial scale, *Z. mobilis* cannot yet compete with yeast, which is very robust when it comes to tolerating acidic conditions. Ethanol production can be carried out under conditions in which lactic acid bacteria are no longer active. Sterilization of the media is not necessary. On the other hand, very costly equipment would be required with *Z. mobilis*, and the energy costs for sterilization of the fermentation broth and the production plant would be considerable. It's a real shame because the rate of ethanol production by *Z. mobilis* is higher than by yeast. On top of that, a final alcohol concentration of 20 percent (volume/volume) can be reached—simply not possible with yeast.

### Okay, but what are the researchers working on?

They are designing a fermentation process and the necessary equipment to make ethanol production with *Z. mobilis* economically feasible. Geneticists have implanted genes into the chromosome of *Z. mobilis* so that it can grow on other substrates, for example, sugars such as pentoses. They are also studying fermentation by immobilized *Z. mobilis* cells. The organisms are attached to small beads in vessels that can be flushed with sugar solutions, speeding up the conversion of sugar into ethanol so that undesired processes such as lactic acid fermentation don't have a chance to take place. Compared to yeast, bacteria have the advantage of being able to ferment not only conventional sugars such as glucose, fructose, or saccharose (from sugar cane or sugar beets) but also cellulose and many other constituents of wood. These microorganisms thus have the potential to convert plant wastes, for example, into ethanol. This is a challenge that requires the use of genetic engineering.

Besides *Z. mobilis*, researchers are concentrating on our commensal *Escherichia coli*. This microorganism is not only able to grow with oxygen and nutrients but also in the absence of oxygen, when it carries out fermentation processes leading primarily to the formation of organic acids and very little ethanol. It was a challenging project to convert *E. coli* into a good ethanol producer. Lonny O. Ingram (Gainesville, Florida, USA) was one of the first to achieve this. The genes for pyruvate decarboxylase and alcohol dehydrogenase from *Z. mobilis* were transferred to *E. coli*, and the pathways leading to unwanted acids were blocked. This resulted in an efficient ethanol producer (Figure 32b). In addition, the spectrum of nutrients taken up and degraded by such a production strain was widened by genetic manipulation. This has opened up a field that may eventually lead to ethanol production strains superior to yeast. We asked Douglas Clark (Berkeley, California, USA) to give us his opinion on the perspectives in this area.

“Lignocellulose is a remarkable material designed by Nature to stand tall and weather the storm in the face of many environmental challenges. It is composed primarily of three materials: cellulose, the most abundant polymer on Earth, hemicellulose, and lignin. These three components

intertwine and reinforce each other to form a very durable composite that requires harsh treatment and/or specialized enzymes for deconstruction. Indeed, deconstructing lignocellulose into monomeric sugars suitable for fermentation into ethanol is a critical bottleneck in the large-scale economic conversion of biomass into biofuel. Fortunately, extremophiles and their enzymes may be of significant help in this regard.

Release of cellulose and hemicellulose from lignocellulose requires pretreatment of the biomass to make it more accessible to enzymatic attack. Once the cellulose is available in a relatively unhindered and less crystalline form, enzymatic hydrolysis by cellulases (or hemicellulases in the case of hemicellulose) proceeds much more quickly. One strategy to lower the cost and improve the efficiency of enzymatic degradation is to hydrolyze cellulose at a relatively high temperature, which would reduce the risk of microbial contamination, facilitate high solids loadings, and possibly provide higher overall reaction rates and greater compatibility of the degradation process with pretreatment conditions. To this end, the isolation of new thermophilic glycosyl hydrolases (e.g., cellulases and xylanases) from thermophiles and even hyperthermophiles is of keen interest. Indeed, cellulases have recently been discovered in bacteria and archaea that inhabit environments where cellulose is apparently in short supply (e.g., deep-sea vents and volcanic hot springs). These enzymes could potentially be used in their native forms or be modified for improved performance by modern tools of protein engineering.

The prevalence of cellulases and related enzymes in thermophiles and other extremophiles remains to be fully determined, but examples of cellulases from extreme environments are beginning to emerge. This is good news for the quest to find enzymes that are better suited to degrade lignocellulose into fermentable sugars under conditions that meet the requirements of a commercial process. Likewise, the microbes that produce such enzymes may prove useful for the fermentation itself, although developing an efficient thermophilic production strain for biofuels remains a formidable challenge. Nonetheless, the discovery of cellulolytic bacteria and archaea in unexpected places, along with the enzymes that support their unusual lifestyle, expands the realm of possibilities for producing biofuels from recalcitrant feedstocks.”

A process leading from lignocelluloses to ethanol would be an important step forward.

#### **By the way, how much bioethanol is currently being produced?**

As already mentioned, yeast is still the workhorse of bioethanol production. The volume produced in 2008 was 65 billion liters (17.3 billion gallons), 52 percent of this in the USA and 37 percent in Brazil. The principal substrate for ethanol production in the United States is corn starch, which is enzymatically hydrolyzed to

glucose and then fermented; in Brazil, it is sugar extracted from sugar cane. Those 65 billion liters really are a lot, but this is only 4.6 percent of the 1.4 trillion ( $1.4 \times 10^{12}$ ) liters of liquid fuel consumed annually.

It is interesting to compare the volume of bioethanol production with the ethanol present in the beer and wine produced globally. Around 131 billion liters (34 billion gallons) of beer are produced (and consumed!) annually, and 25.7 billion liters (6.8 billion gallons) of wine. For a rough estimation, let's assume that beer contains an average of 5 percent ethanol and wine, 10 percent. These beverages then contain approximately 9 billion liters of ethanol, only 14 percent of the bioethanol produced industrially. When compared, these figures also document the gigantic volume of gasoline produced from oil.

Everybody knows that beer and wine taste very different: the sources of fermentable sugars differ as well as the ingredients responsible for taste. Beer brewing goes back to the Sumerians, the original inhabitants of Babylonia, the land between the Tigris and Euphrates Rivers. Their recipe for making beer is documented on the famous clay tablet dating from 7000 BC on display at the Louvre in Paris. The Sumerians were masters at growing cereal grains, including barley, and they learned early that germinated barley grains resulted in a broth suitable for fermentation. Now we know the reason: germination activates an amylase, which then hydrolyzes the starch present. This is the prerequisite for brewing because yeast is able to degrade the sugars glucose or maltose, but not starch. Maltose, which consist of two glucose moieties, and glucose are present in the broth as the result of amylase activity. The Sumerians also must have known how to handle fire because, after saccharification of the barley starch, the broth was boiled to precipitate proteins and cell debris. The resulting liquid, naturally contaminated with yeast, was then fermented, whereas nowadays, yeast cultures are added.

The growing of grapes and wine making were already recorded in the Bible. The history of wine making is connected with Mediterranean cultures, and it spread throughout the Roman Empire. Later, the Christian faith along with its foundation of monasteries as well as European imperialism during the Middle Ages played important roles in the development of the culture of wine production. A key prerequisite for making a good wine is the quality of the grapes. However, the soil, the way the grapes are cultivated, and the regional climate are also important factors affecting the quality of wine. Once the grapes have a sufficient sugar content, they are harvested and collected in a vat, where they are crushed. The juice produced is then placed in wooden casks or stainless steel tanks. Fermentation is allowed to proceed using the yeast naturally present on the grapes or, as with beer, by addition of special yeast cultures. After a year or so, the wine is bottled and stored for a period of time, depending on the kind of wine.

Bioethanol in the form of pulque, mead, beer, wine, and many other alcoholic beverages has accompanied mankind for millennia. A century ago, the application of bioethanol emerged as an important commodity in the chemical industry and, especially now, as a fuel to at least partially replace oil-based gasoline. But it has to be kept in mind what was said in Chapter 14: a further increase of bioethanol production may not be at the expense of our forests and of sufficient food for the world's population. This will be discussed further in Chapter 17.



It is dangerous, says Voltaire, to be right in matters  
where established men are wrong

Georg-Christoph Lichtenberg

## Chapter 17

### Energy conservation from renewable resources

The term “energy production,” frequently used in connection with microbes, is incorrect. According to the law of conservation of energy, energy cannot be produced in a closed system, but it can be converted from one form to another. To use a “microbial example,” part of the energy present in glucose can be converted to the energy present in methane, then into heat when the methane is burned.

What mankind increasingly needs are energy carriers for provision of heat and electricity, for mobility, and for production of goods. The most suitable energy carriers are those with a high energy density. Liquids and gases, or electrical power, are favored for ease of transport. The volume of the energy carriers required globally in 2003 amounted to 15.3 billion tons of coal equivalents (TCE); by 2030 it is expected to increase by 52 percent to 23.3 billion TCE. The proportion of renewable energy carriers was 13 percent in 2003 and will probably reach 14 percent by 2030. In view of this relatively small percentage of renewable energy sources, it is apparent that coal, oil, gas, and nuclear materials will continue to be the predominant energy sources in the near future.

#### Isn't that a rather conservative point of view? Can't we expect a boost in the use of renewables?

Let's look into renewables without regarding the use of wind, water, and solar energy. Instead, let's concentrate on biological processes. Whenever the use of biomass is being considered, the question of energy density is of increasing importance due to transportation costs. Microorganisms are therefore put to work to convert biomass into liquid or gaseous energy carriers. For example, liquid carriers are ethanol and butanol; gaseous carriers are methane (biogas) and molecular hydrogen. The industrial ethanol production with baker's yeast, *Saccharomyces cerevisiae*, is fully established. In addition, genetically engineered strains of *Escherichia coli* have been developed (see Chapter 16). Butanol is of interest as an energy carrier and, even more so, as a basic chemical for industry (see Chapter 15). However, the major drawback of the acetone-butanol fermentation is the low butanol yield of only a few percent, which makes recovery of butanol quite costly.

Whereas the production of ethanol and butanol requires sophisticated technical equipment and engineered strains of yeast, *E. coli* and *C. acetobutylicum*, the

production of biogas is comparatively simple. Large vessels called anaerobic digesters are filled with biomass, and the microbial degradation process begins. First, any oxygen present is consumed, then fermentations leading to products such as organic acids and alcohols take place. Eventually the substrates for methanogenesis emerge: acetate, methanol,  $H_2$ , and  $CO_2$ . Then the methanoarchaea take over and produce biogas. The biomass composition has an effect on how much methane there is in the biogas produced. Biogas produced from sugars and carbohydrates consists of approximately 50 percent methane and 50 percent carbon dioxide. When a certain amount of fat is present, the methane yield is higher than 50 percent. As already mentioned, industrial plants for biogas production are rather simple. They can be built to different scales, with small ones to provide biogas for households or large ones to process all the activated sludge continuously produced in sewage plants of large cities. India is famous for its millions of small biogas plants that provide energy for cooking, especially in rural areas. Bioenergy villages have been developed, but there are certain limitations. Bioenergy cities are an illusion because biomass collection and transportation would simply use too much energy in the form of vehicle fuels.

It has been calculated that half the biomass produced on agricultural areas would have to be channeled into the biofuel fermentation industry in order to meet the annual global need for fuel. There we face the problem already mentioned in a previous chapter. Biofuel production must be kept in balance with global food production and may not be carried out at the expense of long-lived biomass.

Ethanol, butanol, and biogas—these will be the major products of the fermentation industry in the future, but it still will be difficult to meet more than 10 percent of global fuel needs with this type of bioenergy. These processes and some new developments were summarized in a report by the American Academy for Microbiology (Microbial Energy Conversion, 2006).

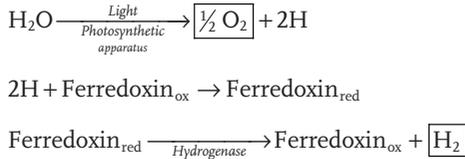
### What about biodiesel?

Products recovered from oil plants, such as rapeseed and soybean oils, cannot be used directly as biodiesel. Such oils consist of long-chain fatty acids that are linked to glycerol. A chemical process has to be performed by which methanol replaces the glycerol, so methanol is required for biodiesel production. Glycerol is not only a byproduct of this process but also an important commodity for biotechnology (see Chapter 28). The biodiesel produced is a valuable component of the so-called energy mix but, again, only a small percentage of petroleum-based fuels can be replaced by biodiesel from rapeseed or related plants.

### What other biological systems can be used for generation of useful energy carriers?

One of the most interesting processes is using solar energy for cleavage of water to molecular hydrogen and molecular oxygen. In fact, that is what plants and cyanobacteria do (see Chapter 9). They are capable of cleaving water with the help of photosystem II. The molecular oxygen produced in this process is released. The

two hydrogen atoms (2H) of water, however, are not converted into molecular hydrogen (H<sub>2</sub>) but transferred to carrier molecules, which then serve as hydrogen donors for conversion of carbon dioxide into starch or other cellular constituents. In order to produce O<sub>2</sub> as well as H<sub>2</sub> from water in light-dependent reactions, additional enzyme systems are required that are able to evolve H<sub>2</sub> like the hydrogenases. If the components, photosystem II, ferredoxin as hydrogen carrier, and a suitable hydrogenase, were to be combined, then water could indeed be cleaved in a light-dependent reaction:



### That's it!

Unfortunately, this works only in principle. Such a system would only function for a few minutes, mainly because the oxygen formed is so reactive that it would inactivate the whole system (see Chapter 5, "Oxygen is a nasty stuff"). The question is how plants manage to cope with this radical action of oxygen. They have developed a protective system in which the O<sub>2</sub> reacts with a target molecule called D1 protein, which is damaged in the process. A repair mechanism continually replaces damaged D1 protein. It's like having to change the spark plugs of a car every five minutes. Obviously, driving a car under these conditions would not be very convenient and, for similar reasons, the enzyme system explained above would not be suitable for H<sub>2</sub> production. An additional drawback is the oxygen sensitivity of most hydrogenases, which also would be inactivated. This whole field finds broad interest and the research required is nearly unlimited. The sunlight-driven cleavage of water into hydrogen and oxygen would eventually lead to a hydrogen-powered economy. In our opinion, this is the only process of global importance in which biological systems or chemical systems mimicking biological processes could be employed on a large scale for generation of energy carriers. The other energy carriers mentioned operate essentially on relatively narrow roads, whereas what we need is a broad new avenue. Together with endeavors using solar energy in photovoltaic plants or in plants using solar energy to heat liquids, these processes will put the sun at the hub of energy-conservation technologies. And that is exactly what we have to do.