Hast thou not poured me out as milk, and curdled me like cheese?

Job 10:10

Chapter 18
Cheese and vinegar

In many ways the existence of mankind is associated with milk, not only breast milk but also cow, sheep, goat, or camel milk products. These products, with an annual global consumption of around 670 million tons, are mainly produced by lactic acid fermentation. The social and cultural development of mankind has strongly been influenced by this fermentation, which facilitated the conservation and storage of food, a prerequisite for development of human settlements. Furthermore, lactic acid fermentation is part of the basic metabolism of muscles, without of course the participation of bacteria, and it is of vital importance in the intestinal tract of infants (see Chapter 10).

If raw milk is allowed to stand at room temperature, sour milk will be formed after about 10 hours. The naturally present population of lactic acid bacteria multiplies by fermenting the lactose present in the milk. The resulting lactic acid causes the principle milk protein, casein, to coagulate at a pH value of around 4.6. This reaction leads to curdled milk products such as curds and various types of cheese. Most cheeses, for example, Emmental, Gruyère, Cheddar, or Gouda, are produced in a slightly different process. First, the milk undergoes a mild lactic acid fermentation that does not coagulate the casein. Instead, coagulation is caused by addition of what is called lab-ferment, or rennin (chymosin). This enzyme from calf stomachs, now also produced by genetically engineered microorganisms, splits a certain bond in the casein molecule. This alters the solubility of casein, so it begins to coagulate at a pH value of 5.5. In the cheesery, the coagulated casein, or curd, is pressed to separate it from the liquid whey. The curd then undergoes a maturation process that finally results in the various types of cheese, such as goat's cheese (Figure 33).

And how is yogurt produced?

The production of yogurt also follows the same pattern but begins with milk thickened by heating. Then a mild lactic acid fermentation followed by soft coagulation gives a product with the consistency typical of yogurt. The lactic acid bacteria used today to make such products include *Streptococcus thermophilus*, *Lactobacillus bulgaricus*, and *Streptococcus cremoris*. Dairies and cheeseries no longer rely on the milk's natural population of lactic acid bacteria; instead, so-called starter cultures

Discover the World of Microbes: Bacteria, Archaea, and Viruses, First Edition. Gerhard Gottschalk.

Figure 33 Making goat cheese: (a) the starter culture is added to the warm goat milk; (b) micrograph of the starter culture; (c) the enzyme chymosin (rennin) is added; (d) after coagulation of casein, a "cheese harp" is used to cut the mash so the whey can be drained; (e) the whey is sucked off; (f) the raw cheese is put into sieve forms; (g) cheese after ripening in a salt brine for three days. (Photographs: Anne Kemmling; cheese dairy in Landolfshausen, Lower Saxony, Germany.)

are used. These cultures are grown in special laboratories, tested for activity and safety, then made available to users. The use of these starter cultures allows such high-density bacterial growth in the milk that any undesirable bacteria are unable to compete against them.

Of course, milk is not the only substrate for lactic acid bacteria. Another product of these bacteria is sauerkraut, which is made from cabbage in a similar process, but leaves behind the cellulose fibers that cannot be fermented by lactic acid bacteria.

Where do the holes come from in some kinds of cheese?

These holes are actually bubbles in uncut cheese. Towards the end of the coagulation step, a special culture of propionic acid bacteria is added to the raw cheese. Propionic acid production makes a decisive contribution to the aroma and flavor of such cheeses. The fermentation carried out by propionic acid bacteria proceeds according to the following equation:

$$3 \text{ lactic acid} \rightarrow 2 \text{ propionic acid} + 1 \text{ acetic acid} + \text{carbon dioxide}$$

As the raw cheese thickens, the carbon dioxide is unable to escape, so it is trapped in the form of the bubbles mentioned above.

Not only Job referred to curdled milk in a simile, but also Homer, who wrote in the fifth song of the Iliad,

> "Just as fig juice added quickly to white milk clots it at once as it's stirred, that's how fast headstrong Ares healed."

In Germany, "alles Essig" (it's all vinegar) is an old expression meaning that something went wrong or the results are not as expected. Nowadays, this expression unfortunately has largely been replaced by more drastic ones. Many wine drinkers have had the experience of opening the bottle, expecting to inhale the wine's wonderful bouquet, only to realize after the first sip that "it's vinegar!" That there is a connection between wine turning to vinegar and its exposure to oxygen was observed long ago.

Wine connoisseurs may notice crystals that often collect at the bottom of wine bottles, known as "wine diamonds." They consist of the potassium/hydrogen salt of tartaric acid, potassium hydrogen tartrate. A related compound, sodium ammonium tartrate, is of historical importance. While inspecting crystal samples of this salt, Louis Pasteur noticed that they came in two asymmetric forms. These forms are now called D- and L-forms, or R- and S-forms. The theory behind this observation was first developed by Jacobus Henricus van't Hoff (1852–1911), who described the tetrahedron model of carbon, having realized that a carbon atom with different substituents can exist in two stereoisomeric forms. It is interesting that nature mainly consists of one type of stereoisomer. Amino acids, the constituents of proteins synthesized in the ribosomal protein factories, all belong to the L series. However, in bacteria there are a few amino acids of the D series that have special functions. For example, the cell wall of bacteria contains D-alanine, whereas only L-alanine is found in proteins.

After this excursion into stereoisomers, we will now focus on vinegar, which has been valued since ancient times for its flavor and as a food preservative. In order to make the best out of early observations how wine turns to vinegar, wine and other alcoholic solutions were purposely exposed to oxygen. The Orléans process was thus developed, in which alcoholic solutions were exposed to air in large pans or vats. Within a few days, a mat termed as *Mycoderma aceti*, formed on the surface. In the 1870s, this layer was found to contain bacteria, which since

then have been called acetic acid bacteria. Today we know that one species of these bacteria, *Acetobacter xylinum*, is able to form cellulose threads that help form this mat-like layer. Bacteria are able to make all kinds of polymeric compounds, but it is quite rare that bacterial species are capable of making cellulose.

Acetic acid bacteria, so to speak, settle in the environment they "prefer," at the liquid surface of the pans and vats mentioned above. There, they have access to the nutrient ethanol from below and to oxygen from above, which they require for oxidation of ethanol to acetic acid. An expert would describe this process as incomplete oxidation. The alcohol is not completely respired to CO_2; instead, respiration stops at the level of acetic acid. Some acetic acid bacteria initially produce acetic acid, but once the alcohol has been consumed or a high concentration of acetic acid is reached, the latter is again taken up and completely respired to CO_2. Of course, this is not what the producers of vinegar have in mind, so they have to interrupt the process once the concentration of acetic acid has reached its optimum, around 10 percent (volume/volume).

The process of incomplete oxidation is also of ecological interest. Acetic acid bacteria live in a niche characterized by an excess of nutrients. They draw on nearly unlimited resources and, in a sense, avoid the cumbersome process of completely metabolizing the nutrients. Instead, they oxidize them incompletely. Such niches are found on the surface of juices or fruit. Relatives of the ethanol-oxidizing acetic acid bacteria often settle in such an environment. They follow the same strategy, for example, by oxidizing glucose to gluconic acid or glycerol to dihydroxyacetone; the latter is an active component of self-bronzers. The synthesis of vitamin C also includes a reaction step carried out by incomplete oxidizers. Hermann Sahm (Juelich, Germany) has intensively studied these processes, which he tells us about:

> "When today more than 100 000 tons of vitamin C per year can be produced cheaply; this is because of the bacterium *Gluconobacter oxydans*, which belongs to the group of aerobic acetic acid bacteria. *Gluconobacter* has a large number of membrane-associated enzymes that efficiently metabolize various sugars and sugar alcohols by incomplete oxidation. This is the reason why this bacterium has already been used since 1934 for oxidation of the sugar alcohol D-sorbitol to L-sorbose for vitamin C production. After this biotechnological-chemical vitamin C synthesis has successfully been carried out for decades, efforts are being made to develop a purely biotechnological procedure for the production of vitamin C."

The classical process is outlined in the Study Guide. Here, we have asked an expert, Karl Sanford (Palo Alto, California, USA), to summarize the development of an innovative biological process for vitamin C synthesis:

> "Modern-day biotechnology provides a means of gathering all of the relevant genes required to convert sugars, such as glucose and fructose that come from renewable feedstocks, e.g., cornstarch, into ascorbic acid within

a single microorganism. By having all the enzymatic steps in a single microorganism, the environmental sustainability and economic features are improved. Fewer manufacturing steps mean that fewer chemicals, less solvents, and less energy are used to make each pound of ascorbic acid.

The history for this process development is a long one, starting initially with Genentech and Lubrizol in the 1980s. The technology was subsequently licensed to Genencor, who then organized a consortium of companies and the United States Department of Energy's Argonne National Laboratory to work on improving the economics and process designs. This substantial effort started in the mid 1990s and ended in the early part of this century. This work was funded in part by the Advanced Technology Program of the United States Department of Commerce as a vanguard to technologies for conversion of renewable feedstock to chemicals using continuous biocatalysis. Much of this work anticipated the subsequent developments in biofuel production from renewable feedstock using sustainable manufacturing processes and the broader industrial or white biotechnology commercial developments. A focus of this work was the development of continuous biocatalytic reactors for the production of chemicals from renewable feedstocks with ascorbic acid as a first product.

Ascorbic acid was selling for between15 and 20 US dollars per kilogram in the mid-1990s. This pricing was used as a benchmark to design a process that would be cost-competitive or, even better, cost-advantaged relative to the state-of-the-art at that time. However, globalization of the chemical industry was beginning; within a few years, the price had collapsed to just a few US dollars per kilogram and there were more than twenty new ascorbic acid producers. Most of these new entrants were in China, using 'old' technology with a different economic model. Although the Genencor process became a very low-cost process to make ascorbic acid as well as other types of related sugar/acid products such as erythorbic acid, the less-appealing commercial opportunity has kept this process from being put into large-scale manufacturing. With recent prices of ascorbic acid rebounding into the range between 15 and 20 US dollars per kilogram, maybe the time is ripe for reconsideration. Certainly, there is a world-class ascorbic acid process based on exemplary metabolic pathway engineering in a *Pantoea citrea* cell factory."

This is just one example for many biotechnological processes that have been designed and worked out but for economic reasons cannot yet be put into industrial reality.

Now back to vinegar. Of course, its production did not stop at the level of the Orléans process. A faster vinegar process was developed in the 1820s, in which the alcoholic solution passes through a reactor loosely filled with beechwood chips. From below, air flows upward past the alcoholic solution that trickles down through the reactor. Before long, acetic acid bacteria settle on the beechwood chips,

Figure 34 A glance into the vinegar plant of the company "Essig Kuehne KG" in Hamburg (Germany). The huge wooden reactors are made of oak. (Courtesy of the company.)

where they then fulfill their task just as they did in the *Mycoderma aceti* mats mentioned above. All modern processes for production of vinegar are more or less advanced versions of this so-called Schuetzenbach process. In Figure 34 you can see such a plant for vinegar production operated by the "Essig Kuehne" company in Hamburg, Germany, until a few years ago: beautiful wooden vats filled with beechwood chips. This form of biotechnological practice is even pleasing to the eye. Submerged fermentation processes have also been developed. These processes use large, aerated bioreactors of stainless steel in which alcoholic solutions, acetic acid bacteria, and air bubbles are continuously kept in contact by stirring.

We should mention once more the fermenting microorganisms that live in the absence of oxygen and those that are incomplete oxidizers, especially their diverse habitats. When sugary solutions such as fruit juice or milk are left to stand, the dissolved oxygen is quickly respired by aerobically living bacteria. As soon as the oxygen is gone, fermentation processes begin. These lead to formation of large amounts of lactic acid, alcohol, or other fermentation products. A different habitat develops at the interface between the fermented solution and the air. Such a habitat is often dominated by incomplete oxidizers.