ROLE OF BACTERIA IN FERMENTATION

What is Fermentation ? Fermentation is the chemical transformation of organic substances into simpler compounds by the action of enzymes, complex organic catalysts, which are produced by microorganisms such as molds, yeasts, or bacteria. Enzymes act by hydrolysis, a process of breaking down or predigesting complex organic molecules to form smaller (and in the case of foods, more easily digestible) compounds and nutrients. For example, the enzyme protease breaks down huge protein molecules first into polypeptides and peptides, then into numerous amino acids, which are readily assimilated by the body. The enzyme amylase works on carbohydrates, reducing starches and complex sugars to simple sugars. And the enzyme lipase hydrolyzes complex fat molecules into simpler free fatty acids. These are but three of the more important enzymes. There are thousands more, both inside and outside of our bodies. In some fermentations, important by-products such as alcohol or various gases are also produced. The word "fermentation" is derived from the Latin meaning "to boil," since the bubbling and foaming of early fermenting beverages seemed closely akin to boiling.

Fermented foods often have numerous advantages over the raw materials from which they are made. As applied to soyfoods, fermentation not only makes the end product more digestible, it can also create improved (in many cases meatlike) flavor and texture, appearance and aroma, synthesize vitamins, destroy or mask undesirable or beany flavors, reduce or eliminate carbohydrates believed to cause flatulence, decrease the required cooking time, and increase storage life. Most fermentations are activated by either molds, yeasts, or bacteria, working singularly or together. The great majority of these microorganisms come from a relatively small number of genera; roughly eight genera of molds, five of yeasts, and six of bacteria. An even smaller number are used to make fermented soyfoods: the molds are *Aspergillus* , *Rhizopus* , *Mucor* , *Actinomucor* , and *Neurospora* species; the yeasts are *Saccharomyces* species; and the bacteria are *Bacillus* and *Pediococcus* species plus any or all of the species used to make fermented milk products. Molds and yeasts belong to the fungus kingdom, the study of which is called mycology. Fungi are as distinct from true plants as they are from animals. The study of all microorganisms is called microbiology. While microorganisms are the most intimate friends of the food industry, they are also its ceaseless adversaries. They have long been used to make foods and beverages, yet they can also cause them to spoil. When used wisely and creatively, however, microorganisms are an unexploitable working class, whose very nature is to labor tirelessly day and night, never striking or complaining, ceaselessly providing human beings with new foods. Like human beings, but unlike plants, microorganisms cannot make carbohydrates from carbon dioxide, water, and sunlight. They need a substrate to feed and grow on. The fermented foods they make are created incidentally as they live and grow.

Human beings are known to have made fermented foods since Neolithic times. The earliest types were beer, wine, and leavened bread (made primarily by yeasts) and cheeses (made by bacteria and molds). These were soon followed by East Asian fermented foods, yogurt and other fermented milk products, pickles, sauerkraut, vinegar (soured wine), butter, and a host of traditional alcoholic beverages. More recently molds have been used in industrial fermentation to make vitamins B-2 (riboflavin) and B-12, textured protein products (from *Fusarium* and *Rhizopus* in Europe) antibiotics (such as penicillin), citric acid, and gluconic acid. Bacteria are now used to make the amino acids lysine and glutamic acid. Single-celled protein foods such as nutritional yeast and microalgae (spirulina, chlorella) are also made in modern industrial fermentations.

For early societies, the transformation of basic food materials into fermented foods was a mystery and a miracle, for they had no idea what caused the usually sudden, dramatic, and welcomed transformation. Some societies attributed this to divine intervention; the Egyptians praised Osiris for the brewing of beer and the Greeks established Bacchus as the god of wine. Likewise, at many early Japanese miso and shoyu breweries, a small shrine occupied a central place and was bowed to daily. In ancient times fermentation joined smoking, drying, and freezing as basic and widely practiced food preservation techniques. Wang and Hesseltine (1979) note that "Probably the first fermentation were discovered accidentally when salt was incorporated with the food material, and the salt selected certain harmless microorganisms that fermented the product to give a nutritious and acceptable food." The process was taken a step further by the early Chinese who first inoculated with the basic foods with molds, which created enzymes; in salt-fermented soyfoods such as miso, soy sauce, soy nuggets, and fermented tofu, these aided salt-tolerant yeasts and bacteria??.

A Brief History of Fermentation in the West. The origins of microbiology (other than the general knowledge of fermented foods which existed worldwide since ancient times) can be traced back to the invention of the compound microscope in the late 1500s. This relatively simple tool soon revolutionized man's knowledge of the heretofore invisible microbial world. In 1675 the Dutch merchant Anton van Leeuwenhoek, the greatest of the early microscopists, saw and reported one-celled organisms, which he called "animacules." (Today they are called "protozoa.") The discovery electrified the scientific world of the time. Then in 1680, using a microscope that magnified the diameter of each object 300-fold, he looked at yeast and found them to consist of tiny spheroids. While the protozoa were clearly alive, the yeast did not appear to be. No connection was drawn between the existence of these tiny organisms and the well known phenomenon of fermentation. So for 150 years after van Leeuwenhoek's pioneering observations, it was hardly thought that these minute organisms could be important enough to deserve serious study.

The early 1800s saw a great increase of interest in microbiology in Europe. The scientific period began with great advances in botany, increased interest in microscopy, and willingness to investigate individual organisms. The two major problems that would challenge the greatest researchers in the new field of microbiology concerned the basic nature of the fermentation process and the basic nature of enzymes. The scientific breakthroughs that would lead to the unraveling of the mysteries of fermentation starting in the 1830s were made primarily by French and German chemists. In the late 1700s Lavoisier showed that in the process of transforming sugar to alcohol and carbon dioxide (as in wine), the weight of the former that was consumed in the process equaled the weight of the latter produced. The first solid evidence of the living nature of yeast appeared between 1837 and 1838 when three publications appeared by C. Cagniard de la Tour, T. Swann, and F. Kuetzing, each of whom independently concluded as a result of microscopic investigations that yeast was a living organism that reproduced by budding. The word "yeast," it should be noted, traces its origins back to the Sanskrit word meaning "boiling." It was perhaps because wine, beer, and bread were each basic foods in Europe, that most of the early studies on fermentation were done on yeasts, with which they were made.

The view that fermentation was a process initiated by living organisms soon aroused fierce criticism from the finest chemists of the day, especially Justus von Liebig, J.J. Berzelius, and Friedrich Woehler. This view seemed to give new life to the waning mystical philosophy of vitalism, which they had worked so hard to defeat. Proponents of vitalism held that the functions of living organisms were due to a vital principal distinct from physico-chemical forces, that the processes of life were not explicable by the laws of physics and chemistry alone, and that life was in some part self determining. As we shall soon see, the vitalists played a key role in debate on the nature of fermentation. A long battle ensued, and while it was gradually recognized that yeast was a living organism, its exact function in fermentations remained a matter of controversy. The chemists still maintained that fermentation was due to catalytic action or molecular vibrations.

The debate was finally brought to an end by the great French chemist Louis Pasteur (1822-1895) who, during the 1850s and 1860s, in a series of classic investigations, proved conclusively that fermentation was initiated by living organisms. In 1857 Pasteur showed that lactic acid fermentation is caused by living organisms. In 1860 he demonstrated that bacteria cause souring in milk, a process formerly thought to be merely a chemical change, and his work in identifying the role of microorganisms in food spoilage led to the process of pasteurization. In 1877, working to improve the French brewing industry, Pasteur published his famous paper on fermentation, *Etudes sur la Biere* , which was translated into English in 1879 as *Studies on Fermentation* . He defined fermentation (incorrectly) as "Life without air," but correctly showed specific types of microorganisms cause specific types of fermentations and specific end products. In 1877 the era of modern medical bacteriology began when Koch (a German physician; 1843-1910) and Pasteur showed that the anthrax bacillus caused the infectious disease anthrax. This epic discovery led in 1880 to Pasteur's general germ theory of infectious disease, which postulated for the first time that each such disease was caused by a specific microorganism. Koch also made the very significant discovery of a method for isolating microorganisms in pure culture.

Interestingly, until his death in 1873, the eminent German chemist J. von Liebig continued to attack Pasteur's work on fermentation, putrefaction, and infectious diseases. He recognized the similarity of these phenomena but refused to believe that living organisms were the main causative agents. Fermentation, he felt, was primarily a chemical rather than a biological process. History has shown, with the discovery of enzymes, that Pasteur was not entirely right, nor Liebig entirely wrong.

The work of Pasteur and his many colleagues and predecessors opened up vast new vistas in the fields of biochemistry, microbiology, and fermentation. The term "biochemistry" was first used in English in 1869, but this new science of the application of chemistry to biology was generally called "physiological chemistry" until the early 1900s. The two outstanding pioneers were Liebig and Pasteur. The term "microbiology" was first used in English in 1885, long after Pasteur's major discoveries. But basic knowledge of this new science of the study of minute living organisms closely related to human activity or welfare did not begin to enter the popular consciousness until the early 1900s. At about this time the scientific breakthroughs of the 1870s and 1880s had begun to produce a change in people's conception of the world around them so sweeping and profound as to be termed revolutionary. Food microbiology was finally set on a scientific foundation, based on the action of specific microorganisms. A rational theory of infectious diseases (which formerly were not differentiated from one another) set people's minds free from the age-old fear of vengeance from an unknowable and invisible disease-causing entity. And the ancient theory of spontaneous generation of lower life forms, which said they could arise *de novo* and fully formed from decomposing matter, was replaced by the verifiable theory of biogenesis. For the first time people began to accept the fact that they shared their environment with multitudes of minute organisms that exerted an ongoing powerful influence on human life. This new world view, among other things, provided a tremendous stimulus for new research on fermented foods.

Although showing that fermentation was generally the result of the action of living microorganisms was an epic breakthrough, it did not explain the basic nature of the fermentation process, or prove that it was caused by the microorganisms that were apparently always present. As early as the late 1700s it had been recognized that there was another type of chemical change that resembled the yeast fermentation in some respects. This was the sort of changes that occur, for example, in the digestion of food. In 1752 Reamur, in studying the digestive processes of a falcon, showed that its digestive juices were able to dissolve meat. In 1785 William Irvine discovered that aqueous extracts of sprouted barley caused liquefaction of starch. The first clear recognition of what were later called "enzymes" came in 1833 when two French chemists, A. Payen and J.F. Persoz, made a more detailed investigation of the process of solubilizing starch with a malt extract to form a sugar that they called "maltose." They called the agent responsible for this transformation "diastase" and they showed that it was destroyed or inactivated by boiling, that without undergoing permanent change itself, a small amount of diastase could convert a large amount of starch to sugar, and that it could be concentrated and purified by precipitation with alcohol. In 1835 the German naturalist Swann, mentioned above for his early work with fermentation, isolated a substance from gastric juice which could bring about the dissolution of meat but which was not an acid. He called it "pepsin" from a Greek word meaning "digestion." It soon became fashionable to call organic catalysts such as diastase and pepsin "ferments," because digestion and fermentation, both allied with life, seemed to be somewhat similar processes. Under the influence of the vitalists, ferments were grouped into two types: those involved with life process were called "organized ferments" and those which were not (like pepsin) were merely "unorganized ferments." A relation between the two types of ferments was suspected by many, and in 1858 M. Traube put forward the theory that all fermentations were due to ferments, definite chemical substances he regarded as related to the proteins and produced in the cells by the organism. In 1876, to reduce confusion that existed concerning the two types of ferments, the German physiologist Wilhelm Kuehne suggested that an unorganized ferment, acting in the absence of life, be called an "enzym," after the Greek words meaning "in yeast;' in 1881 this term was anglicized to "enzyme" by William Roberts, and it had begun to catch on by the 1890s.

Many scientists, including Pasteur, had attempted unsuccessfully to extract the fermentation enzyme from yeast. Success came finally in 1897 when the German chemist Eduard Buechner ground up yeast, extracted a juice from them, then found to his amazement that this "dead" liquid would ferment a sugar solution, forming carbon dioxide and alcohol . . . just like living yeasts. Clearly the so-called "unorganized ferments" behaved just the organized ones. From that time on the term "enzyme" came to be applied to all ferments. The term "ferment" dropped out of the scientific vocabulary altogether and the vitalist position collapsed, never to recover. Thereafter it was agreed that only one set of laws applied to all things, both animate and inanimate, and that there was no special vital force which characterized living things and acted under different laws. And it was finally understood that fermentation is caused by enzymes which are produced by microorganisms. In 1907 Buechner won the Nobel Prize in chemistry for his work, which opened a new era in enzyme and fermentation studies.

The sciences of microbiology, biochemistry, fermentation technology, mycology, and bacteriology all shared a deep interest in the nature and working of enzymes. Yet still by the early 1900s no one knew exactly what enzymes were or how they acted. As the agricultural microbiologist Conn asked in 1901, "How can they produce chemical actions without being acted upon or entering into the reactions? Are enzymes fully lifeless or semi-living? We still do not know the fundamental mystery of fermentation." Gradually an understanding of enzymes and catalysts developed. In 1905 Harden and Young discovered coenzymes, agents necessary for the action of enzymes. In 1926 the American biochemist J.B. Sumner first purified and crystallized an enzyme (urease) and showed that it was a protein, more precisely a protein catalyst. Eventually enzymes came to be seen as the key catalysts in all the life processes, each highly specialized in its catalytic action and generally responsible for only one small step in complex, multi-step biochemical reactions. Enzymes are still produced only by living organisms, both animals and plants; they have never? been synthesized.

Advances in microbiology and fermentation technology have continued steadily up until the present. For example, in the late 1930s it was discovered that microorganisms could be mutated with physical and chemical treatments to be higher yielding, faster growing, tolerant of less oxygen, and able to use a more concentrated medium. Strain selection and hybridization developed as well, affecting most modern food fermentations (Hesseltine and Wang 1977).

A Brief History of Fermentation in East Asia . Traditional fermented foods play an unusually extensive role in East Asia food systems. These fermented foods have a number of important distinguishing characteristics: a number of the most important fermentations use molds; dairy products and other animal proteins (excepting fish) are not widely used, as they are in the West; and modern fermentation processes and technology are based largely on traditional processes, yet are extremely advanced and sophisticated.

The main use of molds has been in the process of making koji (mold-fermented grains and/or soybeans), which serves as a source of more than 50 enzymes in a subsequent fermentation in much the same way that, in the West, the enzymes of malt (steeped and sprouted barley or other cereal grains) are used to make alcoholic beverages.

Since ancient times the koji making process has been unique to East Asia, where it has been used in the preparation of fermented foods such as miso, soy sauce, soy nuggets, sake, shochu (spirits), and rice vinegar (*yonezu*). The only traditional East Asian fermented soyfood not prepared with molds is Japan's natto, and its relatives *thua-nao* in Thailand and *kinema* in Nepal; these are bacterial fermentations. Some have suggested that molds are widely used since they grow well in areas having a humid climate and long rainy season during the warm months. In the West mold fermented foods are limited primarily to a number of cheeses characterized by their strong flavors and aromas: Camembert, Blue, Brie, and related types. Because of the widespread use of mold-fermented foods in East Asia, the word "mold" there has a rather positive connotation, something like "yeast" in the West. Most Westerners still have a deep-seated prejudice against moldy products, and they generally associate the word "mold" with food spoilage, as in "moldy bread."

Surprisingly little has been published in English about the history of fermentation and knowledge of the fermentation process in East Asia, especially the history prior to the 1870s and 1880s, when the new science of microbiology was introduced from the West. The few works that do exist will be cited later.

The earliest records of the koji-making process can be traced back to at least 300 BC in China and to the third century AD in Japan. Molds differ in one important respect from yeasts and bacteria in that they can be easily observed with the naked eye (without a microscope) and their growth, form, and color noted. In East Asia it was probably understood that fermentation was a life process long before it was in the West. By the sixth century AD, as recorded in the *Ch'i-min yao-shu* (the earliest encyclopedia of agriculture), the Chinese had distinct names for two types of molds used in fermented soyfoods; what we now call *Aspergillus* was then called "yellow robe" and *Rhizopus* was called "white robe." These cultures were carefully distinguished and propagated from year to year. By the 10th century a koji starter or inoculum was deliberately being used in the preparation of koji for fermented foods (Tamiya 1958; Sakaguchi 1972; 1979).

From these early times until the 1870s the traditional fermented foods industries in East Asia apparently advanced largely by an empirical, trial-and-error process without the benefit of general scientific research into the nature of microorganisms and of the fermentation process, and without any general theories in these areas.

Prior to 1870, makers of East Asian fermented foods were unaware of the basic nature of the fermentation process of microorganisms, enzymes, and their respective interactions. Makers of koji had no idea what caused the grains and/or soybeans to become covered with a fragrant white mycelium after several days of incubation in a warm koji room, or what later transformed the koji almost magically into delicious, savory seasonings such as miso, shoyu, or soy nuggets, or into heady beverages such as sake.

The advances in food fermentations resulting from the exchange of people and ideas was most pronounced in Japan. The first generation of European scientists there plunged in to their investigations of the many fermented foods with great curiosity and enthusiasm. One of their first subjects of research was the koji mold, now known as *Aspergillus oryzae*, and the various foods in which it was used, especially sake and shoyu, which were major sources of tax revenue for the Meiji government. Tradition ascribed the introduction of sake brewing in Japan to some emigrants from Korea at about the end of the third century AD; they doubtless learned the process from China, where it had long been practiced. One of the earliest accounts of sake production by a Westerner appeared in 1874 when Dr. J.J. Hoffmann, a German professor in the medical school of today's Tokyo University, published a translation of an article on sake from a Japanese encyclopedia of 1714. In 1884 Ferdinand J. Cohn, a Polish botanist and microbiologist, first gave the koji mold its present name, *Aspergillus oryzae* . After 1884 the koji mold was referred to as *Aspergillus oryzae* (Ahlburg) Cohn, in recognition of Ahlburg's earliest accurate description.

Another pioneer in the field of koji research was Atkinson, who had a BS degree from London and was a professor of analytical and applied chemistry at Tokyo University. In 1878, after visiting sake factories, he wrote "On Sake Brewing," which contained a preliminary description of the koji-making process and mentioned the word "koji." In 1881, after extensive research with his assistant Mr. Nakazawa at the koji plant of Mr. J. Kameyama in Yushima near Tokyo, he published two major articles. In his 73-page "On the Chemistry of Sake Brewing," he gave a detailed account of koji making in underground caves in Tokyo and an analysis of its composition.

Another early leader in the fields of microbiology and fermented soyfoods was K. Saito. He did excellent early investigations on the shoyu fermentation, named the primary tempeh mold (*Rhizopus oligosporus*) in 1905, and was an authority on yeasts and molds. Likewise K.N. Yabe did important early work in bacteriology and in natto fermentation.

Two other early pioneers in the introduction of microbiology and fermentation science to Japan were Dr. Teizo Takahashi (1875-1952) and his student Dr. Kinichiro Sakaguchi (1897-), both of whom were professors in the Department of Agricultural Chemistry of Tokyo University. Both men did numerous important studies relating to miso, shoyu, and the koji mold, *Aspergillus*.

During the 20th century, Japanese microbiologists have made many important contributions to the development of applied and industrial microbiology, including the manufacture of fermented soyfoods, as well summarized by Tamiya (1958) and Sakaguchi (1972). Until quite recently, their strength was more in the area of application of scientific knowledge than in pioneering basic scientific and microbiological breakthroughs. From the early 1900s, important studies on the koji mold and its enzymes were done by Japanese scientists. Important advances in enzymology, with much of the work done on koji molds, began in the 1920s. In 1928 Miyazaki developed the combined Amylo-Koji process. By the 1950s Japanese scientists had isolated various protease and amylase enzymes, induced mutations, and used them commercially. They also developed the technology for the microbial production of L-glutamic acid and monosodium glutamate (MSG), lysine and other amino acids, flavor enhancing nucleotides such as inosinic acid, and organic acids. They used the koji mold *Aspergillus oryzae* in the commercial production of enzymes including proteases, amylases, amyloglucosidase, and lipase. They made microbial rennet and numerous other products. Indeed in the period following World War II, Japan became the world leader in the field of industrial fermentations. Wang and Hesseltine (1979) have suggested that this may have been "in large part due to the food fermentation base from which it launched its industrialization of micoorganisms."