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Chapter 5

Food Biotechnology

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5.1 History of Food Biotechnology

Biotechnology has been broadly defined as the utilization of biologically derived molecules, structures, cells, or organisms to carry out a specific process (Wasserman *et al.*, 1988). Many conventional food processing techniques utilize living organisms and bioactive

molecules especially in the brewing and fermentation industries. Alcoholic food and drinks, cheese, yogurt, lactic acid fermented vegetables, soybean sauce, and fish sauce have been made for thousands of years by using naturally occurring microorganisms that grow in a specific environmental condition. Plant enzymes such as malt have been used in the brewing industry long before man acquired the knowledge of enzyme chemistry.

Traditional food fermentation technologies are based on the natural process whereby wet foodstuff undergoes microbial degradation. When it is edible, we call it fermented food, and when it is not, we call it spoiled or putrid food. Man has acquired fermentation skills over a long time and has developed unique technologies suitable for the specific environment and raw materials available in different regions of the world. The first product of fermentation man discovered was alcoholic fermented fruits, which contain sugar that is fermented by natural yeast to make alcohol. More sophisticated fermentation skills using cereals to make alcohol were developed later: beer in Egypt and rice wine in Northeast Asia, both around 4000 BC (Owades, 1992; Lee, 2001). The oldest known written recipe was found on a 4000-year-old Mesopotamian clay tablet. The recipe was for beer (Owades, 1992). The Babylonians made 16 kinds of beer using barley, wheat, and honey. The Chinese book *Shijing* (1100–600 BC) has a poem describing a “thousand wines of Yao,” a legendary nation of China around 2300 BC. It appears that the fermentation technology in Northeast Asia must have been invented by the littoral foragers of the primitive Pottery Age (8000–3000 BC) before agriculture began (Lee and Kim, 2016).

Drying and fermentation were the most important food preservation technologies until the industrial revolution in the seventeenth century in most regions of the globe from temperate to tropical regions. The world of microorganisms was opened to human beings with the invention of the microscope by Antonie van Leeuwenhoek (1632–1723), and the scientific control of fermentation began with the studies of Louis Pasteur (1822–1895). Pasteur showed that good wine batches had certain types of ferment (microorganism), and bad batches had other types of ferment. By heating juices at 63 °C for 30 minutes he could kill the bad ferments, and after cooling the juice he could consistently produce satisfactory wine by inoculating ferments from good wine batches into the juice. This idea was also applied in the pasteurization process of milk, which contributed greatly to the improvement of food hygiene.

Enzymes, the biocatalysts, have been known since the early seventeenth century from observation of their role in digestion and fermentation processes. However, isolation of the crystalline form of enzyme was first achieved with urease in 1926. Thereafter amylase, carboxypeptidase, papain, and pepsin were isolated from plants, animals, and microorganisms. With the development of enzyme technology, the conventional industrial enzymes originating from plants and animals were substituted by microbial enzymes. Chymotrypsin from microorganisms containing the milk clotting enzyme chymosin has partially replaced rennet from the stomach lining of young calves. When the advent of genetic engineering techniques developed in the 1970s, the first application of

genetically modified organism (GMO) in food was the production of food enzymes. Using GMO microorganisms, numerous food enzymes have been developed that have higher activity and are more tolerant of extreme working conditions such as high temperature. The production of biotech crops, especially corn and soybean, has increased rapidly since their first marketing in 1995. The cultivation area of genetically modified (GM) crops reached over 181 million hectares in a total of 28 countries in 2014 (James, 2014). Table 5.1 summarizes the milestone events of food biotechnology through history.

5.2 Traditional Fermentation Technology

The traditional fermented foods of the world can be classified by the materials obtained from bioconversion, such as alcohol fermentation, acid fermentation, carbon dioxide (bread) fermentation, and amino acid/peptide fermentation from protein (Steinkraus, 1993; Lee, 2001). Depending on the raw materials used numerous varieties of fermented food are made in each class of fermentation (Steinkraus, 1983). For example, in alcohol fermentation the following drinks are made: wine from grape, cider from apple, toddies from palm sap, beer from barley or corn, *chongju* from rice and even *mayuchu* from horse milk. Furthermore, by distillation, brandy, rum, vodka, whisky, and soju are produced. Figure 5.1 shows a traditional fermented food map of the world.

The traditional societies can be divided by the indigenous fermented foods they produce; for example, the cheese/yogurt culture of the Middle East, Northern Africa, and Europe, the fish sauce culture of Southeast Asia, and the soybean sauce culture of Northeast Asia. These products are made by the breakdown of proteins to produce the umami (meaty) taste of amino acids and peptides, and form the basic flavor of the meals and condiments characterizing the dietary culture of these different regions.

5.2.1 Alcohol Fermentation

Alcoholic beverages have played an important role in human spiritual and cultural life both in Western and Eastern societies. Unlike in Europe and the Middle East, where most of the indigenous alcoholic beverages are produced from fruits, in the Asia-Pacific region alcoholic beverages are produced from cereals and serve as an important source of nutrients. European beer uses barley malt as the major raw material, while Asian alcoholic beverages utilize mold-grown *nuruk* made from rice or wheat as the fermentation starter.

Table 5.1

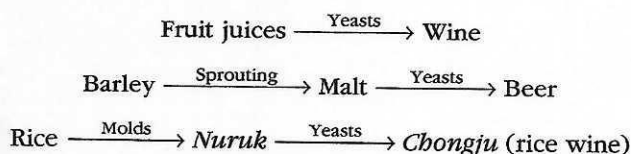
Milestones in food biotechnology.

Date	Milestone in food biotechnology
6000 BC	Use of earthenware for cooking and storage in Northeast Asia Yeasts employed to make wine and beer in the Middle East
4000 BC	Leavened bread produced with the aid of yeasts in Egypt Fungal fermentation of cereals in earthen jars in Northeast Asia Salt fermentation of marine products and plants in earthen jars Curdling of milk in skin bags for cheese making in the Middle East
2000 BC	Fermentation starter 'nuruk' employed for rice wine 'Thousand rice-wines in Yao' of China Use of soybean as food in South Manchuria and the Korean Peninsula
200 BC	<i>Bacillus</i> species employed to ferment soybean 'shi'
1680	Antoni van Leeuwenhoek invented the microscope and discovered microbes
1857	Louis Pasteur discovered anaerobic fermentation Pasteurization process began
1876	Pasteur proved microbial action on beer fermentation
1897	Buchner discovered that enzymes in yeast juice convert sugar into alcohol
1904	Pure cultured fermentation starter 'koji' developed in Japan
1912	Industrial chemicals (acetone, butanol, glycerol) obtained from bacteria
1928	Alexander Fleming discovered penicillin
1953	Industrial production of glutamate by soil bacteria Double helix structure of DNA revealed by Watson and Crick
1960	Industrial enzyme production from microorganisms
1965	Borlaug's Green revolution
1973	DNA recombination by Cohen and Boyer
1975	Hybridomas which make monoclonal antibodies first created
1976	US NIH guidelines on genetic engineering
1982	Genetic engineered insulin approved for use in diabetics in the US and UK First approval for release of GM microbes into the environment
1994	Introduction of GM Flavr Savr Tomato on market by Calgene, Inc. Herbicide-tolerant Round-Up Ready GM soybean by Monsanto Co.
1996	Herbicide-tolerant and insect-resistant YieldGard GM corn on market
2000	Development of Golden Rice™

Alcohol is produced from glucose by the action of the alcohol-producing yeast, *Saccharomyces cerevisiae*. In 1810 Gay-Lussac established the fermentation formula:



The general processes of alcohol fermentation of wine, beer and rice wine are presented as follows:



Malt contains amylases and is able to break down starch into fermentable sugars. The fermentation starter, *nuruk* in Korea, is made by growing molds on cereals, either raw or cooked, to digest starch into sugars, which

are then consumed by the yeast to produce alcohol. Rice-wine fermentation is therefore called a two-step fermentation process. Table 5.2 summarizes the names of cereal fermentation starters used in Asia-Pacific regions, their ingredients, and the microorganisms involved.

5.2.1.1 Wine

Wine is the fermented product of grape, mainly of cultivars of *Vitis vinifera*. Fermented juices of many other fruits, for example apples, berries, peaches, and even herbs, are also called wine. The distinctive character of various wines depends on the composition of the raw material, the nature of the fermentation process, and processing and aging treatments. Table wines with excess carbon dioxide include white, pink, and red sparkling wines, with or without Muscat flavor (champagne, spumante, Sekt, etc.). Non-sparkling white table wines

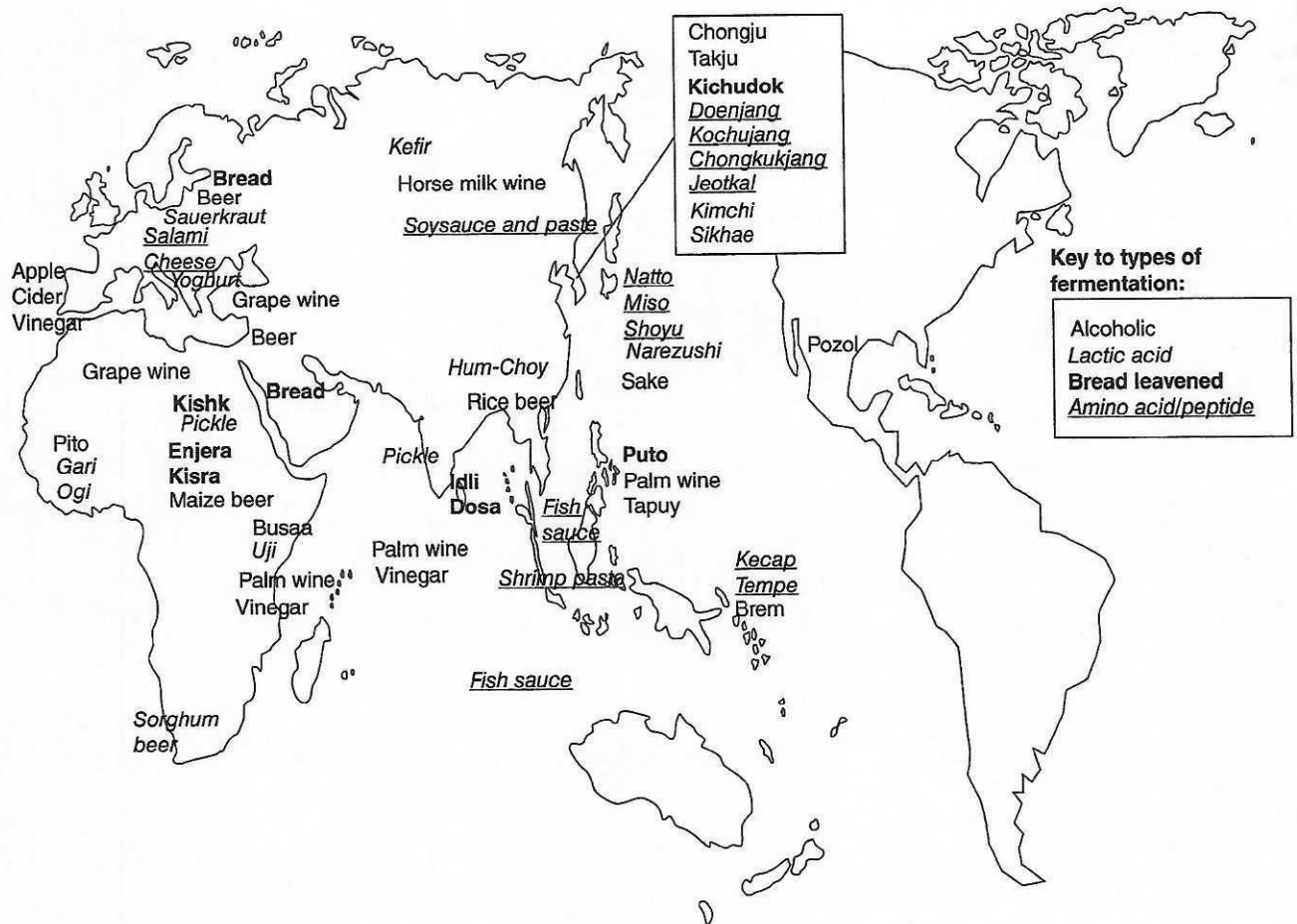


Figure 5.1

Traditional fermented food map of the world.

Table 5.2

Names of fermentation starters in different countries and the major ingredients used (Lee, 1998).

Country	Name	Ingredients commonly used	Shape	Microorganisms
China	Chu	Wheat, barley, millet, rice (whole grain, grits, flour or cake)	Granular	<i>Rhizopus</i> <i>Amylomyces</i>
Korea	Nuruk	Wheat, rice, barley (whole grain, grits or flour)	Large cake	<i>Aspergillus</i> , <i>Rhizopus</i> Yeasts
Japan	Koji	Wheat, rice (whole grain, grits or flour)	Granular	<i>Aspergillus</i>
Indonesia	Ragi	Rice (flour)	Small cake	<i>Amylomyces</i> <i>Endomycopsis</i>
Malaysia	Ragi	Rice (flour)	Small cake	No data available
Philippines	Bubod	Rice, glutinous rice (flour)	Small cake	<i>Mucor</i> , <i>Rhizopus</i> <i>Saccharomyces</i>
Thailand	Loogpang	Bran	Powder	<i>Amylomyces</i> <i>Aspergillus</i>
India	Marchaa	Rice	Flat cake	<i>Hansenula</i> , <i>Mucor</i> <i>Rhizopus</i>

are dry or sweet with regional, varietal, and proprietary names (Riesling, Chardonnay, Chablis, Sauternes, etc.). Pink and red table wines are among the most important in terms of volume or consumer demand and have varietal, regional and proprietary names (Cabernet Sauvignon, Pinot noir, Burgundy, Bordeaux, etc.). There are also numerous dessert wines named after the grape variety from which they are produced, the processes, and the added flavors and herbs (Amerine *et al.*, 1980).

Wines are produced in many different ways. In general, grapes are washed, removed from the stem, and macerated. The must (crushed grapes) is stored in a fermentation vat. The normal flora of ripe grapes and the winery contain sufficient yeast to initiate fermentation. However, the addition of pure actively growing yeast cultures or pressed yeast is common and desirable, and numerous strains of yeast for wine making are available in the market. To prevent growth of undesirable microorganisms 25–100 mg/L sulfur dioxide (in the form of potassium metabisulfite) is added to the must about 2 hours before the yeast. Sulfur dioxide acts as a selective antiseptic for bacteria and wild yeasts, and it permits the more rapid growth of the added yeast.

Wine makers would traditionally allow the natural yeast flora of the grapes to ferment the batch of grape juice, or "must," and some still do this. A variety of yeasts colonize grape skins, with *Kloeckera* and *Hanseniospora* being the most prevalent and *Saccharomyces* present in very low numbers. In contrast, the surfaces of winery equipment generally harbor a yeast flora in which *Saccharomyces* predominates, and these may be the major source of this species. Alternatively, the must to be fermented can also be inoculated with a starter culture of freeze-dried wine yeast to initiate the fermentation. Again, as for beer, the initial stage of the wine-making process is allowed to proceed aerobically in order to develop a sufficient yeast cell population, and then the main fermentation is performed anaerobically to maximize ethanol yield (Aldsworth, 2009).

The strains of yeast used to produce wine commercially are most often *Saccharomyces uvarum* and *S. pastorianus* (Nguyen and Gaillardin, 2005). Both *S. uvarum* and *S. pastorianus* have a greater ethanol tolerance than the beer strains, meaning that the fermentation can proceed for longer and yield a higher concentration of ethanol.

The optimum temperature for fermentation differs in red and white wines; red musts are fermented at up to 26.7°C and whites to 15°C. Red musts are fermented on the skins until the maximum color is extracted, often 5–10 days. The solid material, now called the marc or pomace, is transferred to the press. The fermentation of white musts, at the lower temperature, may last several weeks. A basket press or Willmes press in the larger wineries is often used for the filtration. For fining of the

filtrate, gelatin solution or egg white is added to combine with tannins and related compounds and precipitate. In order to remove excess potassium acid, tartrate wines are stored at a low temperature of approximately –2°C. The clarified wines are bottled and stored in a cool place for aging.

The maximum sugar accumulated by grapes during ripening depends on the variety of grape and the climate of the region where the grapes are grown. For most varieties approximately 15–25% sugar is reached, but late-harvest fruits may have 30–40% sugar. The actual percentage of alcohol in the finished wine depends not only on the sugar content of the must but also on the completeness of the fermentation, and in the case of dessert wines, the amount of alcohol added during or after the fermentation. At least 9 vol % of alcohol is needed to prevent rapid acetification of the finished product. When the sugar content of the grapes is too low to attain this percentage alcohol (1% sugar yields 0.55% alcohol according to the fermentation formula), sugar or grape concentrate may be added. The process, called chaptalization, is normal in cool-climate regions (eastern United States, Germany, France [Burgundy], etc.) but is prohibited in other areas (California, Spain, Italy, etc.).

The production of a sparkling wine such as champagne goes through a very interesting second fermentation process. The wine is initially fermented as for any other wine. However, after the primary fermentation, the wine is transferred to thick-walled pressure-resistant bottles and dosed with a small amount of a yeast and sugar suspension and then given a temporary cap. The dosed wine is inverted at an angle, and the bottles are turned or "riddled" at frequent intervals so that the yeast cells settle toward the cap. When the champagne is judged to be ready, the neck of the bottle is frozen to freeze the plug of cells into it. The cap is then gently trapped to release it, and the pressure of CO₂ within the bottle ejects the plug. After this the bottle is corked and a wire cage applied over the cork to prevent its subsequent unintended ejection (Aldsworth, 2009).

5.2.1.2 Beer

Beer is an alcoholic beverage derived from barley malt, with or without other cereal grains (rice, corn, sorghum, and wheat), and flavored with hops. Malt is made in three steps: the steeping of barley, germination, and drying. By keeping steeped barley (45% moisture) in a humid and dark place for 4–6 days the barley starts to germinate and produce starches, splitting enzymes, α -amylase and β -amylase, and also protease and cellulase. When the modification is completed, the malt is dried. Germination is then terminated by roasting the grains in a kiln; the duration of the roast affects the color and flavor of the malt. A short roast yields a malt of light golden color

and mild flavor, and is used to brew light beers such as lagers, while a longer roast leads to a much darker grain with a much stronger toasted or burnt flavor used to brew stronger beers such as ale, bitter (also called heavy by the Scots), and stout. The malt is coarse milled to form "grist" that can be transported to the brewery and/or stored. The drying stabilizes the malt and allows beer to be produced year round anywhere, even where barley is not grown. This differs from wine, which is only produced seasonally and only near grape-growing areas.

Beer production involves three distinct stages: brewing, fermentation, and finishing. An extract of the crushed malt and the grains selected is prepared to make wort. This step takes about 4–10 hours at around 50°C and is often referred to as brewing. The mashing temperature and duration vary with the mashing systems. Mashing converts insoluble starch into fermentable sugars, maltose and glucose, and proteins into peptides and amino acids. The converted mash is separated into liquid (wort) and insoluble husk by filtration. The clear wort is boiled in a kettle. During the heating period, hops are added. The insoluble humulones in the hops undergo a chemical rearrangement during this process to form isohumulones, which are soluble in water and impart to beer a palate-cleansing bitterness that provides beer with its unusual property of drinkability (Owades, 1992).

The next stage is fermentation, the conversion of wort by yeast into beer. Yeast is added to a cool wort containing oxygen, fermentable sugars, and various nutrients including amino acids. The two main types of beer, lager and ale, are fermented with different strains of yeast. Lager is produced by bottom-fermenting *Saccharomyces uvarum* (*carlsbergensis*) at fermentation temperatures between 7 and 15°C, and at the end of fermentation, these yeasts flocculate and collect at the bottom of the fermenter. For the production of ale, top-fermenting yeast, *Saccharomyces cerevisiae*, is used at fermentation temperatures between 18 and 22°C. *Saccharomyces cerevisiae* is less flocculent and collected for reuse from the surface of the fermenting wort (Russell and Stewart, 1995). The differentiation of lagers and ales on the basis of bottom and top cropping has become less distinct with the advent of vertical conical bottom fermenters and centrifuges.

The fermented beer may be finished in several ways. The simplest and most widely used method is merely to transfer the beer to another tank, chilling it en route, and keeping it for 7–14 days, which is called 'rest.' During this period much of the still suspended yeast settles, and some harsh, sulfury notes and undesirable flavor compounds, notably diacetyl, are removed. After finishing, the beer is filtered, always in the cold, through diatomaceous earth as the filter medium. For sterile filtration to remove all yeast and lactobacilli, another diatomaceous earth filter, cotton fibers, a porous plastic sheet or a ceramic filter

are used as the retaining barrier. If packaged beer has not been sterile-filtered, it must be pasteurized, as beer is a fertile medium for many microbes. The pasteurization may be done just before filling (bulk pasteurization) or after filling in long tunnels with hot-water sprays (tunnel pasteurization). Bulk pasteurization takes about a minute, tunnel pasteurization about an hour.

5.2.1.3 Rice Wine

Rice wine is a generic name for alcoholic beverages made from cereals, mainly rice, in East Asia. Traditional alcoholic beverages vary from crystal-clear products to turbid liquid or thick gruels and pastes. Clear products, which are generally called *shaosingjiu* in China, *chongju* in Korea, and *sake* in Japan, contain around 15% alcohol and are designated as rice wine. Turbid beverages, *takju* in Korea and *tapuy* in the Philippines, contain less than 8% alcohol along with suspended insoluble solids and live yeasts, and are referred to as rice beer. Examples of alcoholic beverages prepared from cereals in the Asia-Pacific region are listed in Table 5.3 (Lee, 2001).

The process of cereal alcohol fermentation using *nuruk* involves two-step fermentation: a solid state fermentation growing mold on raw or cooked cereals, which is called *nuruk*, and mashing the *nuruk* with additional cereals to produce alcohol using yeast. The dried and powdered *nuruk* is mixed with water and stored in a cool place for several days to make a 'mother' brew. During this period the microbial amylases and proteases are activated and convert starches into sugars. The acid-forming bacteria in *nuruk* produce organic acids to bring the pH below 4.5. About two to three volumes of cooked grains and water are added to the mother brew to prepare a first fermentation mash. By the addition of new cooked grains and water to the mash the volume of production increases and the alcohol concentration and quality of the final product enhances. Multiple brews prepared by adding newly cooked grains to the fermenting mash two, three, four, and up to nine times have been described in the old literature (Yoon, 1993).

Newly cooked cereals are added at the end of each step of the fermentation process. The incubation period for each step of the brewing process varies from 2 days to 1 month depending on the fermentation temperature. Low temperatures (about 10°C) are better for improving the taste and maintaining the quality of rice wine. Traditionally rice wines are prepared in late autumn or early spring, when ambient temperatures are below 10°C in the Far East. The volume of wine produced is approximately the same as that of the raw grain used (Rhee *et al.*, 2003).

The traditional method of rice-wine brewing was industrialized by Japanese brewers in the early twentieth century. They adopted pure starter culture, rice *koji* and

Table 5.3

Examples of cereal alcoholic beverages in the Asia-Pacific region (Lee, 2001).

Product	Country	Major ingredients	Microorganisms	Appearance and usage
<i>Rice wine</i>				
Shaosingju	China	Rice	<i>Sac. cerevisiae</i>	Clear liquid
Chongju	Korea	Rice	<i>Sac. cerevisiae</i>	Clear liquid
Sake	Japan	Rice	<i>Sac. sake</i>	Clear liquid
<i>Rice beer</i>				
Takju	Korea	Rice, wheat	Lactic acid bacteria <i>Sac. cerevisiae</i>	Turbid liquid
Tapuy	Philippines	Rice, glutinous rice	<i>Saccharomyces</i> <i>Mucor</i> <i>Rhizopus</i> <i>Aspergillus</i> <i>Leuconostoc</i> <i>Lb. plantarum</i>	Sour, sweet liquid, paste
Brem bali	Indonesia	Glutinous rice	<i>Mucor indicus</i> <i>Candida</i>	Dark brown liquid, alcoholic
<i>Alcoholic rice paste</i>				
Khaomak	Thailand	Glutinous rice	<i>Rhizopus</i> <i>Mucor</i> <i>Saccharomyces</i>	Semi-solid, sweet, alcoholic
Tapai pulut	Malaysia	Glutinous rice	<i>Chlamydomucor</i> <i>Hansenula</i>	Semi-solid, sweet, alcoholic
Tape-kekan	Indonesia	Glutinous rice	<i>Asp. rouxii</i> <i>Saccharomycopsis burtonii</i>	Sweet/sour, alcoholic paste
Lao-chao	China	Rice	<i>Rhizopus</i> <i>Asp. rouxii</i>	Paste
<i>Alcoholic rice seasoning</i>				
Mirin	Japan	Rice, alcohol	<i>Asp. oryzae</i> <i>Asp. usarii</i>	Clear liquid seasoning

manufacturing technology from Europe and transferred it to Korea and China. Industrial production of rice wine uses pure cultured starter, *koji*, by the steaming of polished rice, inoculation of mold, *Aspergillus oryzae* or *kawachii*, and incubation at 25–30°C for 2–3 days. The mother brew is made by mixing *koji*, yeast seed mash and water, followed by incubation for 3–4 days at 20°C. The main brew is made by adding approximately 10 times the volume of cooked rice and water to the mother brew and fermenting for 2–3 weeks. The fermented mash is filtered to obtain clear liquid and aged in a cool place for 1–2 weeks. It is filtered again and then bottled and pasteurized (Rhee *et al.*, 2003).

Rice beers are produced at a higher temperature of fermentation (about 20°C). The fermentation starter powder is mixed with cooked cereals (rice, wheat, barley, or corn) and water, and then incubated at approximately 20°C for 2–3 days, following which it is filtered

through a fine mesh sieve or cloth. These beers are usually prepared by either single or double brew. Cereal beers are abundant in micronutrients, such as vitamin B groups formed during the fermentation, and provide rapid energy supplements with the ethyl alcohol and partially hydrolyzed polysaccharides (Lee, 1999).

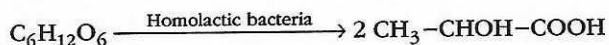
5.2.2 Acid Fermentation

Lactic acid fermentation is probably one of the first biological processes from which human beings discovered the benefits of fermentation (Lee, 1998). The sour ferments of flour dough, milk, cereals, and vegetables have been used for the enhancement of keeping quality and palatability of food from prehistoric times. The fermentation of dairy products in Europe has been widely studied over the past century, and the processes have been

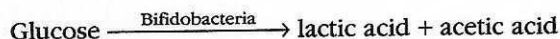
highly standardized and industrialized to ensure efficient production of safe and nutritious food products. Caucasian yogurt and Middle-Eastern cheese have become every-day food for people in Europe, America, and Oceania, and they are considered to be gourmet foods for wealthy people in Asia and Africa. However, little scientific research has been carried out on other types of fermented foods, which have contributed greatly to diets in East Asia and Africa (Lee *et al.*, 1994).

The most important microorganisms for acid fermented food are the lactic acid bacteria, and these are differentiated into four genera: *Streptococcus*, *Pediococcus*, *Lactobacillus*, and *Leuconostoc*. In addition, *Bifidobacterium*, belonging to the order *Actinomycetales*, is also important for dairy products. *Streptococcus*, *Pediococcus*, and some of *Lactobacillus* are homolactic, while *Leuconostoc* and *Bifidobacterium* are heterolactic. The metabolic pathways of glucose in lactic acid bacteria vary: glycolysis, the bifidus pathway, and the 6-P-gluconate pathway.

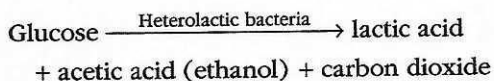
Glycolysis:



Bifidus pathway:



6-P-Gluconate pathway:



5.2.2.1 Lactic Acid Fermented Milk Products

Cultured milk products are produced by the lactic acid fermentation of milk using various bacterial cultures. Fermented milk products originated in the Near East and then spread to parts of southern and eastern Europe. Today cultured milk products in various forms have been introduced throughout the world even in regions where milk is not a traditional food, like Korea and Japan. There are great differences in cultured products depending on the variations in the starter cultures used and manufacturing principles. However, most culture products use the following basic manufacturing steps:

1. culture of starter preparation;
2. treatment of product, such as pasteurization, separation and homogenization;
3. inoculation with bacterial cultures;
4. incubation;
5. agitation and cooling; and
6. packaging.

Table 5.4 lists the world's principal cultured milk products including type, location, and bacterial culture used (McGregor, 1992).

Yogurt is manufactured from milk using *Lactobacillus delbrueckii* ssp. *delbrueckii* and *Streptococcus thermophilus*. Both of these species ferment lactose in the milk to lactic acid as the major end-product. Small amounts of minor components, which are important for flavor, are also evolved with acetaldehyde, diacetyl, and acetone being the most important (Aldsworth, 2009).

Although both *Lactobacillus* and *Streptococcus* are capable of fermenting lactose to lactic acid, milk is quite limiting in the levels of other nutrients that are required by these species. Consequently, a pure culture of either *Lactobacillus* or *Streptococcus* in milk will not yield such a significant, not rapid, pH change as occurs with a mixed fermentation of both species. This has implications for both the quality and the safety of the yogurt made, since gelation will not be so great and the pH may not be reduced enough to inhibit the growth of enteric pathogens such as salmonella.

When inoculated together into milk, streptococci initiate fermentation producing some lactic acid from lactose and also formic acid and CO₂. However, *Streptococcus thermophilus* lacks proteases and so cannot acquire a sufficient supply of nitrogen for vigorous growth, although it does produce peptidases that help it to hydrolyze the limiting concentrations of peptides present in the milk. The lactobacilli do not grow so vigorously in the very early stages of the fermentation, but are stimulated to grow more rapidly by the formate and CO₂ produced by the streptococci. *Lactobacillus delbrueckii* ssp. *delbrueckii* also produces proteases that can hydrolyze milk protein to peptides that *Streptococcus* can then fully hydrolyze, thus yielding amino acids that can be utilized by both species. In the later stages of fermentation, lactobacillus will predominate and perform most of the fermentation as the pH will decline below that which *Streptococcus* can tolerate. The optimum growth temperature for *Streptococcus* is 39°C, while that for *Lactobacillus* is 45°C. Consequently, yogurt fermentation is normally performed at 40–42°C to accommodate both organisms.

Kefir is a soured, effervescent, and slightly alcoholic milk drink originating from the eastern steppes of Russia (Lopitz-Otsoa *et al.*, 2006). The fermentation is performed by a mixture of microorganisms, both yeasts and bacteria bound together by a polysaccharide called kefiran, that are added to the milk in the form of small pellets or granules called "kefir grains." A wide range of organisms are present in the kefir grains, but the most common are various lactobacilli (e.g., *Lb. kefirifaciens*, *Lb. acidophilus* and *Lb. delbrueckii* ssp. *bulgaricum*) and various yeasts (e.g., *Candida kefir* and *Saccharomyces cerevisiae*). Commercially, kefir is produced by heating milk to 85–95°C for 3–10 minutes, cooling to 22°C

Table 5.4

Examples of cultured milk products (McGregor, 1992).

Product	Location	Bacteria
Acidophilus	Europe, North America	<i>Lactobacillus acidophilus</i> , <i>Bifidobacterium bifidum</i>
Bulgarian buttermilk	Europe	<i>Lactobacillus bulgaricus</i>
Buttermilk	North America, Europe, Middle East, North Africa, Indian subcontinent, Oceania	<i>Lac. lactis</i> subsp. <i>cremoris</i> , <i>Lac. lactis</i> subsp. <i>diacetylactis</i> , <i>Leuconostoc cremoris</i>
Filmjolk	Europe	<i>Lactococcus lactis</i> subsp. <i>cremoris</i> , <i>Lac. lactis</i> , <i>Lac. lactis</i> subsp. <i>diacetylactis</i> , <i>Leuc. cremoris</i> , <i>Alcaligenes viscosus</i> , <i>Geotrichum candidum</i>
Flummery	Europe, South Africa	Naturally present lactic bacteria
Ghee	Indian subcontinent, Middle East, South Africa, Southeast Asia	<i>Streptococcus</i> , <i>Lactobacillus</i> and <i>Leuconostoc</i> sp.
Junket	Europe	<i>Lactococcus</i> and <i>Lactobacillus</i> sp.
Kefir	Middle East, Europe, North Africa	<i>Streptococcus</i> , <i>Lactobacillus</i> and <i>Leuconostoc</i> sp., <i>Candida kefir</i> , <i>Kluyveromyces fragilis</i>
Kishk	North Africa, Middle East, Europe, Indian subcontinent, East Asia	<i>Streptococcus</i> , <i>Lactobacillus</i> and <i>Leuconostoc</i> sp.
Kolatchen	Middle East, Europe	<i>Lac. lactis</i> , <i>Lac. lactis</i> subsp. <i>diacetylactis</i> , <i>Lactococcus lactis</i> subsp. <i>cremoris</i> , <i>Saccharomyces cerevisiae</i>
Koumiss	Europe, Middle East, East Asia	<i>Lac. lactis</i> , <i>Lb. bulgaricus</i> , <i>Candida kefir</i> , <i>Torulopsis</i>
Kurut	North Africa, Middle East, Indian subcontinent, East Asia	<i>Lactobacillus</i> and <i>Lactococcus</i> sp., <i>Saccharomyces lactis</i> , <i>Penicillium</i>
Lassi	Indian subcontinent, East Asia, Middle East, North Africa, South Africa, Europe	<i>S. thermophilus</i> , <i>Lb. bulgaricus</i> , sometimes yeast
Prokhlada	Europe	<i>Streptococcus</i> and <i>Lactobacillus</i> sp.
Sour cream	Europe, North America, Indian subcontinent, Middle East	<i>Lactococcus lactis</i> subsp. <i>cremoris</i> , <i>Lac. lactis</i> subsp. <i>diacetylactis</i>
Yakult	East Asia	<i>Lactobacillus casei</i>
Yogurt	Worldwide	<i>S. thermophilus</i> , <i>Lb. bulgaricus</i>

and adding the grains to about 5% w/v. Fermentation proceeds for 8–12 hours at 22°C, after which the mix is cooled to 8°C and incubated for another 10–12 hours.

5.2.2.2 Lactic Acid Fermented Cereals and Starchy Tubers

Lactic acid fermentation of bread dough improves the keeping quality and flavor of the baked products. It also enhances the palatability of bread made from low-grade flours and underutilized cereals. Acid fermented breads and pancakes are an important staple food for people in Africa and some parts of Europe and Asia (Lee, 1994). Sour bread is a typical German food, and Scandinavian rye bread is highly favored by the Nordic people. The Indian *idli* bread types (*idli*, *dosa*, *dhokla*,

kbaman) are important staple foods of the Indian and Sri Lankan people, and they are consumed three or four times a week at breakfast and supper. *Idli* is a small white acid leavened steamed cake made by bacterial fermentation of a thick batter prepared from rice and dehulled black gram. Similar products are made from rice in the Philippines (*puto*) and in Korea (*kichudok*). *Puto* is made using year-old rice, and the batter is neutralized in the course of fermentation. In Sri Lanka *hopper* is prepared from acid fermented dough made with rice or wheat and coconut water. In hopper fermentation, a very large inoculum of baker's yeast or coconut toddy, which includes acid-producing bacteria, is added. Table 5.5 lists various types of acid-fermented bread, pancakes, and starch materials used in different regions (Lee, 1994).

Table 5.5

Examples of acid-leavened bread and pancakes.

Product	Country	Major ingredients	Microorganisms	Usage
Sourbread	Germany	Wheat	Lactic acid bacteria Yeast	Sandwich bread
Ryebread Idli	Denmark India Sri Lanka	Rye Rice Black gram	Lactic acid bacteria <i>Leuc. mesenteroides</i> <i>Enterococcus faecalis</i>	Sandwich bread Steamed cake
Puto	Philippines	Rice	<i>Leuc. mesenteroides</i> <i>E. faecalis</i>	Steamed cake
Kichudok	Korea	Rice	Yeast	Steamed cake
Enjera	Ethiopia	Tef or other cereals	<i>Leuc. mesenteroides</i> <i>P. cerevisiae</i> <i>Lb. plantarum</i> <i>Sac. cerevisiae</i>	Pancake
Kisra	Sudan	Sorghum Millet	<i>Lactobacillus</i> sp. <i>Acetobacter</i> sp. <i>Sac. cerevisiae</i>	Pancake
Kishk	Egypt	Wheat + milk	<i>Lb. casei</i> <i>Lb. brevis</i> <i>Lb. plantarum</i> <i>Sac. cerevisiae</i>	Dried balls
Hopper	Sri Lanka	Rice + coconut water	Yeast Lactic acid bacteria	Steam-baked pancake

Acid porridges prepared from cereals are eaten in various regions of the world, particularly in Africa, where porridges may represent the basic diet (Table 5.6). Nigerian *ogi*, Kenyan *uji* and Ghanaian *kenkey* are examples of porridges prepared by the acid fermentation of maize, sorghum, millet, or cassava, followed by wet-milling, wet-sieving, and boiling.

Acid fermentation is also used to produce food starches with extended shelf-life, resistance to infectious microorganisms and palatable flavor in different regions

of the world. Nigerian *Gari*, Ethiopian *Kocho*, Chinese mung bean starch, and Mexican *pozol* are important acid fermented starch ingredients used for the preparation of porridges, steamed cakes, pastes, noodles, soups, and drinks (Table 5.7).

Most countries in Asia produce mung bean starch, and mung bean starch noodles are a staple of the Chinese diet. The manufacturing process for mung bean starch involves acidic bacterial fermentation. The mung beans are hydrated by soaking in water inoculated with

Table 5.6

Examples of acid-fermented cereal gruels and non-alcoholic beverages.

Product	Country	Major ingredients	Microorganisms	Usage
Ogi	Nigeria	Maize, sorghum, or millet	<i>Lb. plantarum</i> <i>Corynebacterium</i> sp. <i>Acetobacter</i> Yeast	Sour porridge Baby food Main meal
Uji	Kenya Uganda Tanzania	Maize, sorghum, millet, or cassava flour	<i>Leuc. mesenteroides</i> <i>Lb. plantarum</i>	Sour porridge Main meal
Mahewu	South Africa	Malze + wheat flour	<i>Lac. lactis</i> <i>Lactobacillus</i> sp.	Sour drink 8–10% DM
Hulumur Busa	Sudan Turkey	Red sorghum Rice, millet	<i>Lactobacillus</i> sp. <i>Lactobacillus</i> sp.	Clear drink

Table 5.7

Examples of acid-fermented starch ingredients.

Product	Country	Major ingredients	Microorganisms	Usage
Gari	Nigeria	Cassava	<i>Leuconostoc</i> , <i>Alcaligenes</i> <i>Corynebacterium</i> , <i>Lactobacillus</i>	Staple Cake Porridge
Mungbean starch	China Thailand Korea Japan	Mungbean	<i>Leuc. mesenteroides</i> <i>Lb. casei</i> <i>Lb. cellobiosus</i> <i>Lb. fermenti</i>	Noodle
Khanom-jeen	Thailand	Rice	<i>Lactobacillus</i> sp. <i>Streptococcus</i> sp.	Noodle
Pozol	Mexico	Maize	Lactic acid bacteria <i>Candida</i>	Porridge
Me	Vietnam	Rice	Lactic acid bacteria	Sour food ingredient

12-hour steeped water from a previous fermentation to ensure acidification of the beans. The principal microorganisms found in the steep-water are *Leuconostoc mesenteroides*, *Lactobacillus casei*, *Lb. cellobiosus*, and *Lb. fermentum*. The lactic fermentation, which reduces the pH to about 4.0, protects the beans from spoilage and putrefaction that would otherwise occur in ground bean slurries (Steinkraus, 1983).

Thai rice-noodle, *Khanom Jeen*, is also made from acid fermented raw rice. Soaked rice is drained and fermented for at least 3 days before grinding, and *Lactobacillus* sp. and *Streptococcus* sp. are involved in the acid fermentation.

5.2.2.3 Acid Fermented Vegetables

Acid fermented vegetables are important sources of vitamins and minerals. *Leuconostoc mesenteroides* has been found to be important in the initiation of the fermentation of many vegetables (i.e., cabbages, beets, turnips, cauliflower, green beans, sliced green tomatoes, cucumber, olives, and sugar beet silages). In vegetables, *Leuc. mesenteroides* grows more rapidly and over a wider range of temperatures and salt concentrations than any other lactic acid bacteria. *Leuconostoc mesenteroides* produces carbon dioxide and acids, which quickly lower the pH, thereby inhibiting the development of undesirable microorganisms and the activity of their enzymes, which may soften the vegetables. The carbon dioxide produced replaces air and provides anaerobic conditions favorable for the stabilization of ascorbic acid and the natural color of the vegetables. The growth of *Leuc. mesenteroides* modifies the environment, making it favorable for the growth of other lactic acid bacteria. The high acidity produced by the species and other subsequent lactic acid bacteria inhibits the growth of *Leuc. mesenteroides*. *Leuconostoc mesenteroides* converts

glucose to approximately 45% levorotatory D-lactic acid, 25% carbon dioxide, and 25% acetic acid and ethyl alcohol. Fructose is partially reduced to mannitol and is then readily fermented to yield equimolar quantities of lactic acid and acetic acid. The combination of acids and alcohol is conducive to the formation of esters that impart desirable flavors.

Table 5.8 shows examples of acid-fermented vegetables produced in different regions of the world. The difference between sauerkraut and *kimchi* is the preferred end point of fermentation. The best tasting *kimchi* is attained before overgrowth of *Lb. brevis* and *Lb. plantarum* with an optimal product pH of 4.5. The overgrowth of *Lb. brevis* and *Lb. plantarum* diminishes product quality, but sauerkraut production depends on these organisms. The fermentation is manipulated by the salt concentration and temperature. The optimal range of salt concentration of sauerkraut is 0.7–3.0%, while that of *kimchi* is 3.0–5.0% (Lee, 1994).

Kimchi making is the original Korean method of preserving the fresh and crispy texture of vegetables during the winter when fresh vegetables are not generally available. *Kimchi* has a unique sour, somewhat sweet and carbonated taste and is usually served cold. In this respect *kimchi* differs from sauerkraut, which is acidic in taste, but is served warm. Raw materials for *kimchi* are mainly divided into three groups. Korean cabbage and radish are the major materials, and minor ingredients include garlic, red pepper, green onion, ginger, and salt, and lastly, fermented fishery products and other seasoning agents, which are often optional. Whole (or cut) cabbage is salted with 15% brine for 3–7 hours, washed twice with fresh water, and then drained. Other minor ingredients that are chopped and combined are mixed with the treated cabbage, placed in containers, and tightly sealed. The length of time for completion of the fermentation

Table 5.8

Examples of acid-fermented vegetables produced in different regions of the world.

Product	Country	Major ingredients	Microorganisms	Usage
Sauerkraut	Germany	Cabbage, salt	<i>Lb. plantarum</i> <i>Lb. brevis</i>	Salad Side dish
Kimchi	Korea	Korean cabbage, radish, various vegetables, salt	<i>Leuc. mesenteroides</i> <i>Lb. brevis</i> <i>Lb. plantarum</i>	Salad Side dish
Dhamuoi	Vietnam	Cabbage, various vegetables	<i>Leuc. mesenteroides</i> <i>Lb. plantarum</i>	Salad Side dish
Dakqudong	Thailand	Mustard leaf	<i>Lb. plantarum</i>	Salad Side dish
Burong mustala	Philippines	Mustard	<i>Lb. brevis</i> <i>P. cerevisiae</i>	Salad Side dish

depends on the salt concentration and temperature. Winter *kimchi* is made for 2–3 months to attain optimal taste, while summer *kimchi* is made in 3–4 days at 20°C. The optimum pH and acidity for the best taste is 4.2 and 0.6% (as lactic acid), respectively (Breidt *et al.*, 2013).

To make sauerkraut, the washed and shredded cabbage is stacked in large containers and layered with salt to between 2 and 3% w/w before being covered with a weight that helps to force liquid from the cabbage. The salt acts as an inhibitor for many bacterial species, particularly the enteric pathogens. In addition, the salt causes the excretion of water from the plant cells creating a brine environment of the cabbage leaves. This is sufficient for lactobacilli, which are more halotolerant than many other species, to ferment to lactic acid. The reduced pH of the sauerkraut, along with the high salt concentration, acts to extend the shelf-life of the cabbage significantly.

Sauerkraut is not usually inoculated with a starter culture but instead relies on the natural flora of the fermentation vessels. The species that predominate in sauerkraut production include *Leuconostoc mesenteroides* and *Lactobacillus plantarum*. Due to the low pH and high salt content of sauerkraut, it is relatively free of microbiological problems if made correctly. One pathogen that has occasionally been found to contaminate sauerkraut is *Listeria monocytogenes*, which has greater acid and halotolerance than most enteric pathogens. In addition, yeasts and molds can occasionally cause problems with spoilage, again due to their greater acid and halotolerance (Aldsworth, 2009).

5.2.2.4 Acid Fermented Fish and Meat

The storage life of perishable fish and meats can be extended by acid fermentation with added carbohydrates and salt. In Scandinavian countries most traditional low-salt fermented fish products are transformed into

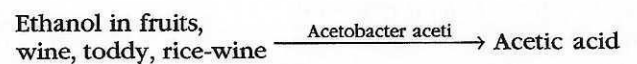
pickled products in vinegar. These products generally require low-temperature storage. On the other hand, most Asian products are lactic fermented with added cereals, as shown in Table 5.9.

Rice, either cooked or roasted, is the most frequently used carbohydrate source, but other sources such as millet in *sikhae* are also used. In some cases fruits and vegetables, for example tamarind in *Bekasam* for the reduction of pH, and garlic and pepper in *sikhae*, are added. The antimicrobial effect of garlic to some putrefactive microorganisms such as *Bacillus* in lactic-fermented fish products has been demonstrated (Souane *et al.*, 1987).

Fermented sausages, salami in Europe, *nham* in Thailand, and *nem-chua* in Vietnam are also made by a process involving lactic acid bacteria. Starter cultures for salami fermentation are isolated from fermented fish products in Korea as well as other Asian countries (Lee, 2001).

5.2.2.5 Vinegar

Vinegar fermentation is as ancient as alcoholic fermentation, since acetic acid is produced in any natural alcoholic fermentation upon exposure to the air.



Vinegars are produced from fruits in Europe, from tropical fruits, such as coconut, sugar cane, and pineapple in the Asia Pacific region and from cereals in North-east Asia. Cereal vinegars may be divided into three classes: rice vinegar, rice-wine filter-cake vinegar, and malt vinegar. The indigenous processes are natural or spontaneous fermentation brought about by the growth of *Acetobacter aceti* on alcoholic substrates under aerobic conditions. Traditionally, degraded or poor quality wines were used for the production of low-grade vinegars

Table 5.9

Examples of acid-fermented seafood and meat products (Lee, 1994).

Product	Country	Major ingredients	Microorganisms	Usage
Sikhae	Korea	Sea water fish, cooked millet, salt, garlic, red pepper powder	<i>Leuc. mesenteroides</i> <i>Lb. plantarum</i>	Side dish
Narezushi	Japan	Sea water fish, cooked millet, salt	<i>Leuc. mesenteroides</i> <i>Lb. plantarum</i>	Side dish
Burong-isda	Philippines	Freshwater fish, rice, salt	<i>Lb. brevis</i> <i>Streptococcus</i> sp.	Side dish
Pla-ra	Thailand	Freshwater fish, salt, roasted rice	<i>Pediococcus</i> sp.	Side dish
Balao-balao	Philippines	Shrimp, rice, salt	<i>Leuc. mesenteroides</i> <i>P. cerevisiae</i>	Condiment
Kungchao	Thailand	Shrimp, salt, sweetened rice	<i>P. cerevisiae</i>	Side dish
Nham	Thailand	Pork, garlic, salt, rice	<i>P. cerevisiae</i> <i>Lb. plantarum</i> <i>Lb. brevis</i>	Pork meat in banana leaves
Sai-krok-prieo	Thailand	Pork, rice, garlic, salt	<i>Lb. plantarum</i> <i>Lb. salivarius</i> <i>P. pentosaccus</i>	Sausage
Nem-chua	Vietnam	Pork, salt, cooked rice	<i>Pediococcus</i> sp. <i>Lactobacillus</i> sp.	Sausage

at the household level. Today, vinegars of high quality standards are produced by industry.

Commercial vinegar is prepared from rice-wine filter-cake in Far Eastern countries. Filter cakes from rice-wine factories are collected and packed tightly into a storage tank for 1–2 years. The filter-cake contains large amounts of unused carbohydrate and proteins, which are further hydrolyzed by inherent microorganisms and enzymes during storage, converting them into alcohol and other nutrients and flavor substances. The cake is slurried in two to three volumes of water prior to filtration. The filtrate is heated to 70°C, and cooled by mixing with fresh vinegar mash to a temperature of 36–38°C, and then fermented with *Acetobacter* for 1–3 months. It is further aged at the ambient temperature for 3–6 months and filtered to obtain clear vinegar (Lee, 2001).

5.2.3 Bread Fermentation

Baking, brewing, and enology all depend on the ability of yeasts to carry out anaerobic fermentation of sugars, yielding CO₂ and ethanol. In brewing and wine making, alcohol is the prime product of interest, while in baking, the leavening effect of CO₂ is more important. Breads are divided into two groups, leavened loaf bread and unleavened flat bread. Traditionally leavening is a result of the products of fermentation, carbon dioxide and ethanol produced by yeast. Although the CO₂-generating chemical leavening agents such as food acid and soda (sodium bicarbonate) can replace yeast, biological leavening imparts

physicochemical modification of dough constituents and flavor development.

Originally sourdoughs were used for the production of all type of breads because commercial baker's yeast was not available. The major microbial species in sourdough fermentations are the lactic acid bacteria (LAB). Fermentation of sourdough by the LAB yields CO₂, which raises the bread its distinctive tangy flavor. Baker's yeast was introduced in the market at the beginning of the twentieth century. Industrially produced yeasts are strains of the top-fermenting species *Saccharomyces cerevisiae* grown on molasses in an aerobic fed-batch fermentation. The optimum temperature for the growth and fermentation of baker's yeast is between 28 and 32°C, and the optimum pH is 4–5. Leavening of dough requires the addition of 1–6% yeast based on the weight of flour. The exact percentage depends on the recipe, the process, and the quality of the flour and yeast as well as the operation conditions (Spicher and Brummer, 1995).

The production of baked goods consists of preparation of raw materials, dough formation (kneading, maturing), dough processing (fermentation and leavening, dividing, molding, and shaping), baking in the oven, and final preparation (slicing, packaging, etc.). Bread dough is fermented for a sufficiently long time to permit the yeast to act on the assimilable carbohydrates and convert them into alcohol and carbon dioxide as the principal end product. By the end of the proofing period, the aqueous phase of the bread is saturated with CO₂ and the volume has roughly doubled as a result

of the pressure of CO₂ that has diffused to air cells. At the beginning of baking, the loaf further expands (oven spring) by the expansion of air and steam during heating, and then at some temperature the matrix sets, expansion stops, and starch gelatinization, crust coloring, and flavor development take place. The magnitude of oven spring depends on two factors: (1) the generation and expansion of gases, and (2) the amount of time available for loaf expansion before the structure sets. The first factor is primarily a function of yeast fermentation; the second factor is affected by dough components such as shortening, surfactants, gluten protein, and flour lipids (Stauffer, 1992).

5.2.4 Amino Acid/Peptide Fermentation

Fermented protein foods are used mainly for flavor-enhancing condiments and gourmet food ingredients due to the meaty and appetite-stimulating flavor of protein hydrolysate, which is formed during the fermentation. The type of indigenous fermented protein food is decided primarily by the availability of the raw material in the specific climatic and geographical conditions. Cheese is made in the Middle East and Europe where animals are the main food source. Fermented soybean products, for example soybean sauce and paste, are used in North-eastern Asian countries, and fermented fish products in the Asia-Pacific region.

5.2.4.1 Cheese

The worldwide number of cheese varieties has been estimated at 500, and there are several methods of classification. Cheeses can be divided by their texture: very hard (Parmesan, Romano), hard (Cheddar, Swiss), semi-soft (Brick, Muenster, blue, Harvarti), soft (Brie, Camembert, feta), and acid (cottage, cream, Ricotta). A broad look at cheeses might divide them into two large categories: ripened and fresh. More technical classifications are also used, for example, those based on coagulating agent: rennet cheese (Cheddar, Brick, Muenster), acid cheese (cottage, Quarg, cream), heat-acid (Ricotta, Sapsago), and concentration-crystallization (Mysost) (Nuath *et al.*, 1992).

Cheese is manufactured by coagulating or curdling milk, stirring and heating the curd, draining off the whey, and collecting or pressing the curd. Characteristic flavor and texture are formed during the ripening of cheese depending on the type of starter culture and microorganisms involved as well as the coagulating agent and salting methods. Depending on the variety, the milk is pasteurized (generally at about 72°C for 16 seconds), and a bacterial starter culture is added to the milk, which is at 30–36°C. The inoculated milk is generally ripened

at the temperature for 30–60 minutes to allow the lactic acid bacteria to multiply sufficiently for their enzyme system to convert lactose to lactic acid. After ripening, a milk-coagulating agent is added. For blue cheese, the mold (*Penicillium* sp.) is added to the starting milk or to the drained curds.

The starter cultures are organisms that ferment lactose to lactic acid and other products. These include *Streptococci*, *Leuconostocs*, *Lactobacilli*, and *Streptococcus thermophilus*. Starter cultures also include *Propionibacteria*, *Brevibacteria*, and mold species of *Penicillium*. The latter organisms are used in conjunction with lactic acid bacteria for a particular characteristic of cheese, for example, the holes in Swiss cheese are due to *Propionibacteria*, and the yellowish color and typical flavor of brick cheese is due to *Brevibacterium linens*.

Coagulation of milk is essential to cheese making. Most proteolytic enzymes can cause milk to coagulate. Rennet (chymosin, EC 3.4.23.4) is widely used for milk coagulation for cheese making. However, due to the shortage of calf's chymosin, commercial rennet may include blends of chymosin and pepsin extracted from the stomach of other animals such as pig. Microbial rennets with similar functionality are also prepared from *Mucor miebei*, *Mucor pusillus*, and *Endothia parasiticus*. Proteases from plants are known to coagulate milk but are not used in commercial cheese making.

Hard cheeses are prepared over the longest period of time and generally have the highest solids and lowest moisture content. For hard cheese varieties, salt is mixed into the curd crumbs and then the curds are pressed in a mold to help expel whey and produce the characteristic wheels or cylinders of cheese. Finally, most hard cheese varieties undergo an extended maturation or ripening stage, during which production of secondary metabolites from endogenous enzymatic activity and also non-starter lactic acid bacterial metabolism can alter the flavor further. In some varieties of cheese, some metabolism of the lactate present by *Propionibacterium freidenreichii* ssp. *sbermanii* to yield propionate, and CO₂ is encouraged as this causes typical holes present in Emmenthal and Gruyere cheese. The curds crumb may not be pressed to mechanically expel whey, but may simply be molded into wheels and allowed to dry naturally. Semi-soft cheeses may finally undergo a shorter maturation process than hard cheeses, but that maturation may be assisted by organisms other than bacteria. For instance, blue cheeses such as Stilton, Roquefort, and Gorgonzola are matured with the aid of the mold strains *Penicillium roqueforti* and *P. glabrum*. Mold-ripened cheeses typically have a higher pH than bacterial-ripened cheese because of the proteolytic activity of the molds and the release of ammonium ion, which buffers the lactate. In addition, the molds initiate lipolysis, leading to a complex mixture of fatty acids that contribute to the pungency of blue

cheeses. Of the bacteria-ripened semi-soft cheeses, some such as Gouda and Edam are actually salted by soaking in brine, rather than mixing salt with the curd crumb. Other bacteria-ripened semi-soft cheeses (e.g., Limburger, Brick, and Port Salut) may be matured by smearing the surface of the cheese with a mixture of bacteria and molds (Deetae *et al.*, 2007). The range of species in the surface smear can be wide (e.g., *Brevibacterium linens*, *Proteus vullgaris*, *Staphylococcus equorm*, and *Geotrichum candidum*) and can lead to some extremely pungent products (Arfi *et al.*, 2003).

On the whole, soft cheeses are manufactured using the same basic protocols and starter cultures as hard and semi-soft cheeses. However, some soft cheeses are not made using a starter culture, but the natural microflora of raw milk is simply permitted to perform the fermentation. An example of this type of cheese would be Mexican queso blanco. However, serious problems with listeriosis in inadequately prepared cheese during the 1980s in southern California have demonstrated the potential problems in making such a high water activity, low salt product from raw milk. The most important feature of the soft cheeses is that the whey is only partially separated from the curds, and in the case of cottage cheese not at all separated. As a result, the water activity for these types is the highest of all the cheese varieties. This results in a reduced shelf-life for such cheeses compared with those that are harder and have a lower water activity (Aldsworth, 2009).

Some soft cheeses may undergo a ripening period. For example, both Camembert and Brie are ripened cheeses and both also make use of mold ripening using species such as *Penicillium camemberti*, *P. caseicolum*, and *G. candidum*. For both Camembert and Brie, the ripening mold grows over the surface of the cheese rather than through it as in Stilton, although the effect of the mold is similar. In both cases, proteolysis occurs leading to increased pH through ammonium release and lipolysis occurs leading to fatty acid release and a concomitant increase in aroma and flavor intensity. The increased pH brought about by mold ripening can lead to problems with spoilage and pathogenic organism survival due to the higher water activity of these cheeses. Some soft cheeses are pickled in brine, for example Feta cheese, while some cheeses such as Mozzarella are not ripened at all. Mozzarella is formed by heating the curd and whey mixture

until the curds form strings. These are then stretched and formed into balls, which are held in the whey until use.

5.2.4.2 Fish Sauce and Paste

Fish fermentation is an old technology used for the preservation of freshwater and marine animals, which are highly perishable, localized in production, and seasonally fluctuating in catch (Ruddle, 1993). The technology appears to have evolved with the availability of salt and a non-pastoral way of life. There is a strong correlation throughout the world between the use of fermented fish products and the use of vegetables and cereals, especially rice (Ishige, 1993). Although the use of fermented fish products is nowadays mainly confined to East and Southeast Asia, traces of this technology can be found throughout old human civilizations.

Aging salted (cured) fish in a container or earthen jar for a longer period produces fish sauce. The enzymes in the gut and from the halophilic microorganisms grown in the system decompose fish meats, and the exuded liquid (protein hydrolysate) is fish sauce. The hydrolysate mainly consists of amino acids and peptides, which form the characteristic meaty flavor of fish sauce. In the case of Korean *Jeotkal* fermentation containing 20% salt, the total number of viable cells increases for the first 40 days, mainly attributed to the growth of *Pediococcus* and *Halo-bacterium*. The concentrations of soluble-N and amino-N increase steadily during the first 60 days, and it coincides with the development of optimum taste. The volatile basic N content increases in two steps, and the second step increase causes the taste deterioration, which is related to the maximum growth of yeast (Lee *et al.*, 1993).

Depending on the amount of salt added, the products are classified as high-salt (>20% salt of total weight), low-salt (6–18% salt), and no-salt products, as shown in Figure 5.2. When the salt concentration is higher than 20% of total weight, pathogenic and putrefactive microorganisms cannot grow and the product does not need other preservative means. The first criterion for the subdivision of this group is the degree of hydrolysis, which is influenced by fermentation time and temperature, added enzyme sources, and water content. The fully hydrolyzed liquid is fish sauce. The term 'cured fish' is confined to represent the partially hydrolyzed fish products that retain the original shape of fish immersed in the

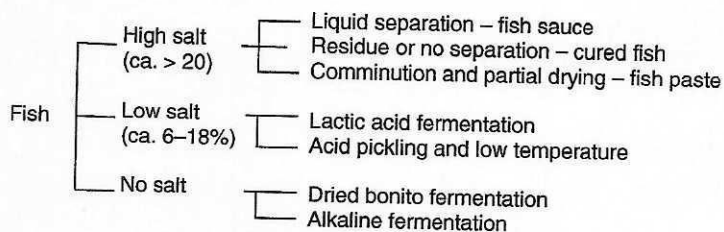


Figure 5.2

Classification of fermented fish products.

exuded liquid, and this form as such is frequently used as a side dish for rice meals. Fish paste is characterized by the salted fish being partially dried in order to restrict the degree of hydrolysis and comminuted to produce the homogeneous, solid condiment. Each class can be further subdivided by the kind of raw materials, such as fish species and portion of fish, and thus, there are numerous kinds of products (Lee, 1989).

Many Asian countries produce salt-cured and dried fish products, for example, *plakem* in Thailand, *jambal-roti* in Indonesia, Maldive fish in Sri Lanka, and *gulbi* in Korea, but the role of fermentation in these products is not fully understood. Fish fermentation without added salt is not a common practice. In some local specialties, half-spoiled fish or alkaline fermentation in leafy plant ash is used. The propagation of mold in dried bonito (*katsuobushi*) processing in Japan is another example of non-salt fish fermentation.

Most countries in East and Southeast Asia have fish sauce, but the flavor, physical properties, and raw materials used vary. Depending on the degree of hydrolysis or fermentation time and the separation method, two types of sauce, namely, clear and turbid, are produced. *Ngan-pya-ye*, *nuoc-man*, *namp-la*, *shottsuru*, and *yu-lu* are clear fish sauces, and *budu*, *patis*, *ketjapikan*, and *jeotkuk* are turbid. Some turbid sauces are from the exuded liquid of cured fish, for example, *patis* from *bagoong* production in the Philippines and *jeotkuk* from *jeotkal* production in Korea. In Northeast Asia, cured fish products are more important than fish pastes. Fish pastes, especially those made from shrimp and planktonic animals such as *Seimsangapy*, *belacan*, *trassi*, *prahoc*, and *kapi* are important in Southeast Asian diets.

5.2.4.3 Fermented Soybean Products

At the early stage of soybean utilization the Northeastern *Dong-yi* probably first invented *shi*, the old Chinese term for Korean *meju*, by keeping cooked soybeans in a pottery jar. Cooked soybeans grown with mold and bacteria, which is called *meju*, are immersed in brine to leach out the protein hydrolysate; the liquid part is soybean sauce (*kanjang*), and the residue is soybean paste (*doenjang*).

The traditional fermented soybean products are divided into three groups based on the type of fermentation starter used: *Shi* made from loose type soybean *meju*, *Maljang* from cake type soybean *meju*, and *Jang* from soybean mixed with other cereals. The propagation of these products in the Northeastern region, namely China, Korea, and Japan, and their variations are shown in Figure 5.3. According to S.W. Lee (1990), fermented soybean products were first introduced to China in the first century BC and to Japan in the sixth century AD. Varieties of products have been developed and have disappeared throughout history.

Korean *kanjang* and *doenjang*

Meju is prepared from cooked soybeans. Soybeans are soaked in water overnight, cooked for 2–3 hours and mashed by pounding. The mash is then shaped like a brick or a ball, dried in the sun, and kept in a stack covered during the night for several days. During this period, the surface grows mold, especially *Aspergillus oryzae*, and the inside grows bacteria, typically *Bacillus subtilis*. The enzymes from mold and bacteria hydrolyze the soybean proteins into amino acids, and the carbohydrates into sugars and organic acids. The amino acids and sugars interact with each other during the browning reaction, resulting in the characteristic dark brown color and meaty flavor. Properly fermented *meju* is immersed in brine in an earthen jar and ripened for several months. The brown color and meaty flavor leach out into the brine. During this period, salt-tolerant yeasts grow in the mash, especially *Saccharomyces rouxii*, which produces the aroma of soy sauce. The liquid part is soy sauce and the precipitates are soybean paste. Soy sauce so produced is boiled once and stored in an earthen jar for years. The flavor of soy sauce gets richer as the storage time increases, just as the flavor of wine becomes smoother as it gets older. It has been said in Korea that the taste of food in a household is decided by the taste of the household's fermented soybean products.

Japanese *shoyu* and *miso*

Japanese people modified the *meju* preparation method in the early twentieth century by controlled fermentation technology using a pure culture of mold isolated from the traditional starter (Shettleff and Aoyaki, 1976). The mold, normally *Aspergillus oryzae*, is grown on cooked rice or cooked wheat grits to make *koji*. It is mixed with cooked soybeans for further fermentation and then ripened in the brine. Soybean paste (*miso*) and soy sauce (*shoyu*) are made separately. For *shoyu*, *koji* is made with cooked defatted soybean flakes and wheat grits and then mixed in brine for aging. After 4–6 months of aging, it is filtered to obtain *shoyu*, the liquid part, and the solid part is discarded. *Miso* is prepared by using *koji* made from cooked rice or other cereals mixed with cooked soybean and salt, and then mashed into a paste and ripened. These processes are easy for industrialization of the products. The flavor of Japanese *shoyu* and *miso* is mild and sweet compared to Korean counterparts. Korean people prefer the strong flavor of traditional soy sauce and soybean paste, the same as European people distinguish Roquefort from processed Cheddar cheese.

Korean *chongkukjang* and Japanese *natto*

Soybeans are cooked and covered with a straw mat or cloth, and placed on the warm stone floor, *ondol*, for 3–4 days until the mucus string is formed. It is mixed with

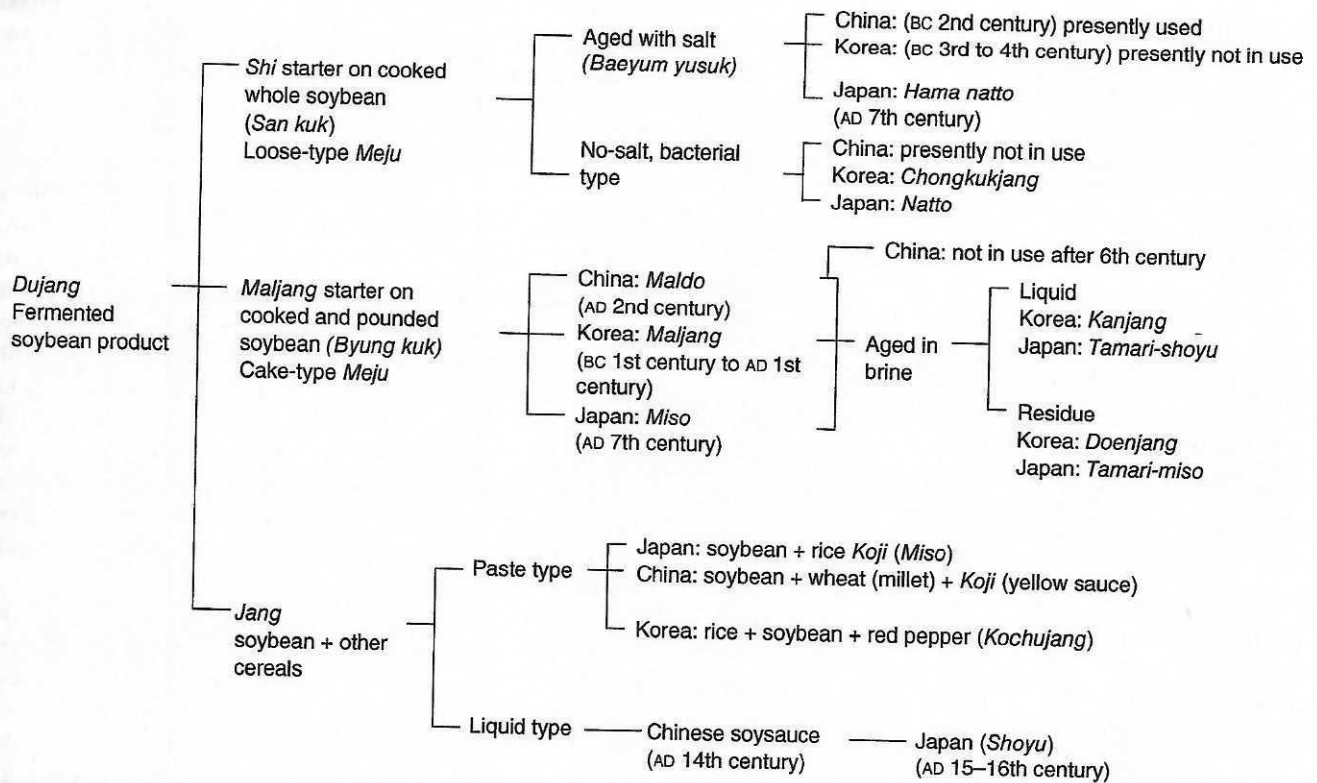


Figure 5.3

The origin and interchange of *Dujang* (fermented soybean products) in East Asia (Lee, 1990).

chopped ginger, chopped garlic, and salt, and pounded slightly until the bean kernels are separated into halves and then stored in an earthen jar. The strong smell of fermented soybean is partially masked by the ginger and garlic smell, and creates the characteristic *chongkukjang* flavor. The spicy seasoning is thus prepared in 3–4 days, while ordinary soybean paste, *doenjang*, which uses *meju* as fermentation starter, takes over 6 months for complete ripening. In this respect, *chongkukjang* is a rapid fermentation method. The mucus substance in *chongkukjang* is peptido-polysaccharide produced by *Bacillus subtilis* (Nout *et al.*, 2014).

Japanese *natto* is a modified form of *chongkukjang*. *Natto* is fermented soybean grown with *Bacillus subtilis* on cooked soybean. The fermented soybean with mucous string is consumed directly without further processing, so it is a non-salt fermented product. However, *natto* is not generally accepted by Korean people. It is always mixed with spices and used for the cooking of vegetable stew as a meaty-flavored condiment. The amount of *chongkukjang* added to the stew is large enough to significantly supplement protein in the diet. *Chongkukjang* was also called *Jeonkukjang* in the old days. 'Chongkuk' means the Chinese kingdom 'Qing,' and 'Jeonkuk' means 'a country at war' or a combat zone. What all these

names imply is that this product was made in extraordinary situations, for example during war time or famine conditions, for the urgent supply of a nutritious savory food ingredient.

Korean kochujang

The basic tastes of European people are sweet, sour, bitter, and salty, and Japanese people add umami, the meaty taste. Korean people add another taste: hot or pungent. The most remarkable difference of Korean food compared to food of neighboring Japan and China is the strong pungent taste of red pepper in most Korean dishes.

Kochujang is a unique hot bean paste seasoning popular in Korea. It is made from fermented soybean starter, *meju*, and malt made from barley. Malt powder is mixed with cereal porridge made from rice, glutinous rice, or barley. The enzymes in malt hydrolyze the starch into sugars and reduce the consistency of the mixture. *Meju* powder, red pepper powder, and salt are added to the partly saccharified porridge with thorough mixing to make a paste, and put in an earthen jar. The top is covered by salt in order to prevent mold growth. The jar is placed in a sunny place for further fermentation. The proteins in soybeans and cereals degrade into amino

acids to produce the meaty flavor. During fermentation a wonderful harmony of the meaty flavor from hydrolyzed proteins and the sweet taste of hydrolyzed starches with the pungent taste of red pepper and salty taste is achieved, and a new characteristic flavor stimulating the appetite of Koreans is formed.

Tempe

Tempe is found in all parts of Indonesia but is particularly important in Java and Bali. It is also produced in some Malaysian villages and in Singapore. *Tempe* is a white mold-covered cake produced by fungal fermentation of dehulled, soaked in water, and partially cooked soybean cotyledons (Steinkraus, 1983). It is packed in wilted banana leaves and sold in the market. Essential steps in the preparation of *tempe* include cleaning the beans, soaking in water, dehulling, and partially cooking the dehulled beans. Dehulling is important for the growth of mold on the surface of cotyledons. The soybeans are not necessarily cooked fully, because subsequent mold growth is able to soften the texture. Under the natural conditions in the tropics, *tempe* production involves two distinct fermentations: bacterial acidification of the beans during soaking and fungal overgrowth of the cooked bean cotyledon by the mold mycelium. A previous batch of sporulated *tempe* or sun-dried pulverized *tempe* powder (1–3 g) is sprinkled over the cooked and drained soybean cotyledon (1 kg) and thoroughly mixed to distribute the mold spores over the surface of all the beans. *Rhizopus oligosporus* is known as *tempe* mold, and the pure culture of strain NRRL2710 or CBS 338.62 can be used for the inoculum.

A handful of inoculated beans are placed on wilted banana leaves or other large leaves and packed. The leaf keeps the soybean cotyledon moist during the fermentation and allows for gaseous exchange. Incubation can be at temperatures from 25 to 37°C. The higher the incubation temperature, the more rapidly the *tempe* molds will grow. For example, 80 hours of incubation is required at 25°C, 26 hours at 28°C, and 22 hours at 37°C. The *tempe* should be harvested as soon as the bean cotyledons have been completely overgrown and knitted into a compact cake. The cotyledons should be soft and pasty (not rubbery), and the pH should have risen to about 6.5 (Nout *et al.*, 2014).

Tempe should be consumed immediately after harvest. It can be stored for 1 or 2 days without refrigeration. If the *tempe* is not going to be consumed immediately, it should be deep-fried, in which form it remains stable for a considerable time, or it should be blanched by steaming and refrigerated. It can be stored after dehydration, either by sun drying or by hot-air drying and kept in plastic bags. Subsequent keeping quality is excellent

because *tempe* contains a strong antioxidant produced by the mold and is resistant to the development of rancidity. *Tempe* is consumed fresh or in deep-fried form.

Chinese sufu

Chinese *sufu* (*tosufu*, *toufuru*, *fuyu* or *taubuyi*) is a highly flavored, creamy bean paste made by overgrowing soybean curd with a mold belonging to the genus *Actinomucor*, *Rhizopus*, or *Mucor* and fermenting the curd in a salt brine/rice wine mixture (Lee and Lee, 2002). In the West, *sufu* has been referred to as Chinese cheese. *Sufu* is usually sold in red or white blocks 2–4 cm² by 1–2 cm thick, and the white *sufu* is untreated, while the red variety is colored with Chinese red rice, *hung chu*. The procedure for making *sufu* consists of five steps: preparation of soybean curd (*tofu*), preparation of molded *tofu* (*pehtze*), salting, fermenting in salt brine/rice wine, and processing and packaging.

Soybeans are cleaned, soaked in water, and ground to make soybean milk slurry. The slurry is heated to boiling and filtered through cloth, and the residue is discarded. Coagulants (calcium chloride/calcium sulfate mixture or sea salt brine) are added to the filtrated soy milk to make soybean curd. The amount of coagulant used to produce *tofu* for *sufu* manufacture is 20% higher than that used for regular *tofu*. Moreover, after the coagulants are mixed with soybean milk, the mixture needs to be agitated vigorously in order to break the coagulated protein into small pieces, after which it is set aside for 10 minutes to complete the process of coagulation. This process reduces the water content of the curd and makes the texture harder. If the water content is more than 60%, the inoculation of fungi is deferred until the water remaining on the curd surface is reduced by drying.

Pehtze is the soybean curd that is overgrown with the grayish hair-like mycelium of molds belonging to genera *Actinomucor*, *Rhizopus*, or *Mucor*. These fungi are normal contaminants in rice straw. Traditionally inoculation was performed by placing the *tofu* on the rice straw, but this method does not always yield a high quality because of undesirable contaminating microorganisms. In spring or autumn when the ambient temperature is 10–20°C, white fungal mycelium is visible on the surface of the cubes after 3–7 days, at which point the cubes are taken out and immediately salted in large earthenware jars. Each layer of *pehtze* is sprinkled with a layer of salt, and after 3–4 days when the salt is absorbed, the *pehtze* is removed, washed with water, and put into another jar for processing (Nout *et al.*, 2014).

For processing, a dressing mixture, which varies for each type of *sufu*, is placed in the jar. To make red *sufu*, *anka koji* and soy mash are added. To make fermented rice (*tsao*) *sufu*, fermented rice mash is added. To make

Kwantung *sufu*, red pepper and anise are added in addition to salt and red *koji*. Alternate layers of *pehtze* and dressing mixture are packed into the jar until it is filled to 80% of its volume, then a brine with a concentration of approximately 20% NaCl is added. Finally, the mouth of the jar is wrapped with the sheath leaves of bamboo shoots and sealed with clay. After 3–6 months fermenting and aging, the *sufu* is ready for consumption (Steinkraus, 1983).

5.2.5 Other Fermented Products

5.2.5.1 Chinese Red Rice (Anka)

Anka, also known as *ang-kak*, *beni-koji* and red rice, is used in China, Taiwan, the Philippines, Thailand, and Indonesia to color foods, including fish, rice wine, red soybean cheese, pickled vegetables, and salted meats (Lee and Lee, 2002). It is a product of fermentation of rice with various strains of *Monascus purpureus* Went. A number of countries are gradually adopting this natural pigment to replace coal-tar dyes that have been implicated as carcinogens. The advantages of using *Anka* are that its raw materials are readily available, the yield is good, the color of pigment produced is consistent and stable, the pigment is water soluble, and there is no evidence of any toxicity or carcinogenicity.

Anka is produced at an industrial scale in Taiwan. Non-glutinous rice (1450 kg) is washed and steamed for 60 minutes. Water (1.8 hectoliter [hL]) is sprayed on the rice, which is again steamed for 30 minutes. The steamed rice is mixed with 32 L of *chu chong tsaw*, a special variety of red rice inoculum in Taiwan, after cooling to 36 °C, and heaped in a bamboo chamber. When the temperature of the rice rises to 42 °C, it is spread on plates and shelved. *Anka* is produced by moistening the rice three times during incubation, followed by final drying; 700 kg of *Anka* is produced from 1450 kg of rice.

An uncommon phenomenon of the mold *Monascus purpureus* is the exudation of granular fluid through the tips of the hyphae. When the culture is still young, the freshly extruded fluid is colorless, but it gradually changes to reddish-yellow and purple-red. The production of the red coloring matter is observed not only on the exuded granular fluid but also on the hyphal content within. The red coloring matter diffuses throughout the substrate. The dark red color consists of two pigments, the red monascorubrin (C₂₂H₂₄O₅) and the yellow monascoflavin (C₁₇H₂₂O₄). The strains of *Monascus purpureus* that are adopted in the production of red rice are only those that are capable of impregnating rice with a dark red color in the presence of water concentrations sufficiently low that no distortion is produced in the hydrated grains.

5.3 Enzyme Technology

The application of enzymes in food processing is an important branch of biotechnology. Enzymes are the proteins that catalyze virtually all chemical reactions occurring in biological systems, and hundreds of enzymes have been identified and characterized. Of importance to the food industry is the production of high fructose corn syrups by using glucose isomerase, saccharification of starch by amylases in baking and brewing, juice clarification by using cellulases and pectinases, production of low lactose milk by using lactase, cheese making by rennin, and meat tenderization by proteases such as papain, bromelain, and ficin. The cell wall degrading enzymes (hemicellulose and cellulase) are used to extract vegetable oil (olive and canola/rape seed) in an aqueous process by liquefying the structural cell wall components of the oil-containing crop. Table 5.10 summarizes some important food enzymes, their source, reaction specificity, and applications in food processing.

In the following section, enzymatic starch modification and protein hydrolysis will be discussed in detail.

5.3.1 Enzymatic Starch Modification

The major steps in the conversion of starch are liquefaction, saccharification, and isomerization. During liquefaction the α -1,4 linkages of amylose and amylopectin are hydrolyzed at random by endo- α -amylase. This reduces the viscosity of the gelatinized starch and increases the dextrose equivalent (DE), a measure of the degree of hydrolysis of the starch. For saccharification to dextrose, a DE of 8–12 is commonly used, and the maximum DE obtainable is about 40 (Olsen, 1995).

β -Amylases are exo-enzymes that attack amylose chains, resulting in production of maltose from the non-reducing end. In the case of amylopectin, the cleavage stops two to three glucose units from the α -1,6 branch points. Isoamylase and pullulanase hydrolyze α -1,6-glucosidic bonds of starch. When amylopectin is treated with pullulanase, linear amylose fragments are obtained.

Maltodextrin (DE 15–25) produced from liquefied starch is commercially valuable for its rheological properties. Maltodextrins are used in the food industry as fillers, stabilizers, thickeners, pastes, and glues. When saccharified by further hydrolysis using amyloglucosidase or fungal α -amylase, a variety of sweeteners can be produced having DE in the range of 40–45 (maltose, 50–55 [high maltose] and 55–70 [high conversion syrup]).

Table 5.10

Some important food enzymes and their usage.

Name	Source	Action mode	Applications
α -Amylase	Malt, <i>Aspergillus</i> , <i>Bacillus</i> spp.	α -1,4 glycosidic linkage of amylase, amylopectin	Starch modification, brewing aid Reduce dough viscosity, prevent staling
β -Amylase	Malt, molds, bacteria	Split off β -maltose from non-reducing end of starch	Production of maltose syrup, brewing and baking aid
Glucoamylase	<i>Aspergillus</i> , <i>Rhizopus</i> spp.	Stepwise hydrolysis of α -1,4 linkages in starch	Production of glucose, analysis of starch content in food
Glucose isomerase	<i>Streptomyces</i> , <i>Actinoplanes</i> , <i>Bacillus</i> spp.	Conversion of glucose to fructose	Production of high-fructose corn syrup, immobilized form is used
Pullulanase	<i>Klebsiella pneumoniae</i>	α -1,6 bond of amylopectin	Production of maltose and maltotrioses, removal of limit dextrins to produce high alcohol beer
Invertase (β -fructo-franosidase)	<i>Saccharomyces</i> , <i>Candida</i> spp.	Hydrolyze sucrose to glucose and fructose	Invert sugar syrup, chocolate coated sucrose candy, recovery of scrap candy, artificial honey, humectants
β -Glucanase	<i>Bacillus subtilis</i> , <i>Asp. niger</i>	β -1,3 or β -1,4 bonds of β -D-glucans	Solubilize barley gums in brewing, reduce viscosity of wort
Cellulase	<i>Trichoderma reesei</i>	Endo-cellulases to split β -1,4 linkage, exo-cellulases, cellobiases	Turn cellulosic waste into glucose to make ethanol, hydrolyze cellulose into β -dextrins and glucose
Pectinases (PG, PL, PE)	<i>Aspergillus</i> spp.	Split glycosidic bonds of pectin, endo/exo	Extraction and clarification of fruit juice
Lactase (β -galactosidase)	<i>Kluyveromyces marxianus</i> , <i>Asp. niger</i>	Hydrolyze lactose into glucose and galactose	Low-lactose dairy products, prevents crystallization of lactose in milk concentrate
Rennet (chymosin, pepsin)	Calf stomach, <i>Endothia parasitica</i> , <i>Mucor meihei</i>	Catalyze k-casein, destabilize casein micelle	Milk clotting in cheese making
Proteases	Plants (papain, ficin), animal (trypsin), <i>Aspergillus niger</i> , <i>Bacillus</i> spp.	Hydrolyze peptide bond esterase activity	Meat tenderizer, chill-proof beer, recovery of scrap meat and fish, gluten modifier, decolorization of red blood cells
Lipases	<i>Mucor</i> , <i>Rhizopus</i> , <i>Aspergillus</i> spp.	Hydrolyze ester linkages of triglycerides	Accelerate cheese ripening, cheese flavor production

5.3.2 Enzymic Modification of Proteins

The enzymic modification of proteins is an attractive means of obtaining better functional and nutritional properties of food proteins. The conversion of milk to cheese is an effect of the action of the protease of the microorganisms inhabiting the system. Enzymatic hydrolysis of milk proteins is used to produce non-allergic and low allergic cow's milk products for baby

food and highly digestible protein foods for hospital patients.

Protein structure is modified to improve solubility, emulsification and foaming properties, gelation, and textural properties. Enzymatic processes provide several advantages compared to chemical treatments, including fast reaction rates in mild conditions with high specificity.

Proteases are classified according to their source of origin (animal, plant, or microbial), their catalytic action

(endo-peptidase or exo-peptidase), and the nature of the catalytic site. Based on a comparison of active sites, catalytic residues, and three-dimensional structures, four major protease families have been recognized to date: the serine, the thiol, the aspartic, and the metallo-proteases. The serin protease family contains two subgroups: the chymotrypsin-like and the subtilisin-like proteases. Many industrially important proteases are mixtures of the different types of proteases. This is especially the case for pancreatin, papain (crude), and some proteases from *Bacillus amyloliquefaciens*, *Aspergillus oryzae*, *Streptomyces* and *Penicillium duponti* (Olsen, 1995).

The degree of hydrolysis (DH) of enzyme-treated proteins determines the properties of relevance to food application. DH is measured by the pH-stat technique (Adler-Nissen, 1986). It is based on the principle that pH is kept constant during hydrolysis by means of automatic titration with a base, when the hydrolyzes are carried out under neutral to alkaline conditions. DH is calculated on the basis of the titration equations as follows:

$$DH = (b/b_{tot}) \times 100\%$$

$$DH = B \times Nb \times 1/a \times 1/MP \times 1/b_{tot} \times 100\%$$

where B is base consumption, Nb is the normality of the base, a is the average of the dissociation of the α -NH₂ groups, MP is the mass of protein, b is the equivalent peptide bonds cleaved per kilogram of protein (or milliequivalents per gram of protein), b_{tot} is the total number of peptide bonds in the protein substrate.

Proteases catalyze the hydrolytic degradation of the peptide chain. When a protease acts on a protein substrate, the catalytic reaction consists of three consecutive reactions:

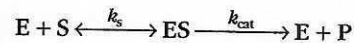
1. formation of the Michaelis complex between the original peptide chain (the substrate) and the enzyme;
2. cleavage of the peptide bond to titrate one of the two resulting peptides; and
3. a nucleophilic attack on the remains of the complex to split off the other peptide and to reconstitute the free enzyme.

The rate-determining step is the acylation step characterized by the reaction velocity constant k_{+2} . Enzymatic hydrolysis of milk proteins and soybean proteins often produces bitter peptides (Kim *et al.*, 2003). The unpleasant bitterness can be managed by proper selection of the reaction parameters and the enzymes used. Bitterness is a complex problem, which can be influenced by many variables, for example, the hydrophobicity of the substrate, since the amino acid side-chains containing

hydrophobic groups become exposed due to the hydrolysis. DH is closely related to the bitterness of the protein hydrolysates.

5.3.3 Enzyme Reaction Kinetics

The activity of an enzyme is determined by many factors, including enzyme, substrate, and cofactor concentrations, ionic strength, pH, and temperature. For conversion of substrate (S) to product (P) by an enzyme (E), the reaction scheme can be simply represented as:



The reaction velocity (V) is then given by the Michaelis-Menten equation:

$$V = \frac{k_{cat}[E][S]}{K_m + [S]}$$

Where K_m is the substrate concentration at which V equals one-half the maximum velocity (V_{max}), as shown in Figure 5.4.

Integration of this equation with respect to time gives:

$$V_{max} = K_m \ln \left(\frac{[S]}{[S_t]} \right) + ([S] - [S_t])$$

Where S and S_t are the substrate concentrations at zero time and time t , respectively. This equation is particularly

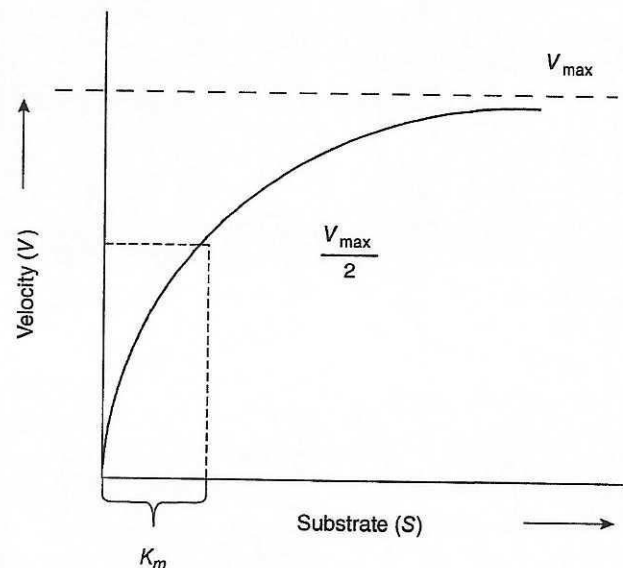


Figure 5.4
Enzyme-catalyzed reaction.

useful in industrial situations where a reaction is allowed to proceed to near completion or equilibrium. K_m and V_{max} can be determined by linearizing the above equation as follows, resulting in a Lineweaver–Burk plot (Karel and Lund, 2003):

$$\frac{1}{V} = \frac{1}{V_{max}} + \frac{K_m}{V_{max}[S]}$$

5.4 Modern Biotechnology

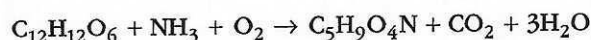
The development of traditional fermentation technology and enzyme technology paved the way for modern biotechnology to achieve industrial mass production of amino acids, nucleic acids, organic acids, and antibiotics in the twentieth century. The production of fine chemicals by fermentation methods has many advantages over chemical synthetic processes. It requires milder, safer, and environmentally friendly reaction conditions, and provides higher productivity and wider varieties of physiologically active substances. Modern biotechnology was initially applied for the mass production of glutamate, the raw material of flavor enhancer, monosodium glutamate (MSG), in the 1950s in Japan, followed by the commercial production of other amino acids, nucleic acid-related substances, and antibiotics. The modern fermentation process is carried out aseptically in the closed fermenter to form useful substances using microbial strains, and converts these to the higher value-added chemicals either by enzymatic or chemical modification by necessity (Figure 5.5). This so-called hybrid technology, combined biotechnology between fermentation and chemical/enzymatic modification, is recognized in both the food and fine chemical industries (Lim, 1999).

5.4.1 Amino Acid Production

Amino acids have many useful functions not only as nutrients but also as preventive pharmaceuticals. The world market for natural L-amino acids is led by glutamate (MSG), lysine, phenylalanine, methionine, and glycine. MSG and feed additive amino acids (lys, met, thr, trp) account for 98% of the market. Phenylalanine is an important raw material for the production of aspartame, the synthetic sweetener.

Glutamate is produced from glucose (industrially raw sugar) through the Embden–Meyerhof Pathway (EMP) and the TCA cycle in the cells of *Corynebacterium glutamicum* or *Brevibacterium lactofermentum* (or *flavum* or *thiogenitalis*), as shown in Figure 5.6.

In order to convert glucose into an amino acid nitrogen source (NH_4^+), energy (NADP) and oxygen are needed:



One mole glucose produces 1 mol glutamate, but in the large fermenter the yield of glutamate is 60–67%.

MSG production involves fermentation, recovery, purification, crystallization, drying, and packaging (Figure 5.7).

Lysine is produced by *Corynebacterium glutamicum* or *Brevibacterium flavum* and their mutants. Aromatic amino acids, mainly phenylalanine, are produced by *Escherichia coli*, *Bacillus subtilis*, and *B. flavum*.

5.4.2 Nucleic Acid Production

Nucleic acid is polynucleotide composed of a pentose (ribose or deoxyribose), phosphorus, and a base unit. Base units are purines (adenine, guanine, and hypoxanthine) and pyrimidines (thiamine, cytosine, and uracil). Among the nucleic acid related substances, 5'-IMP (inosine monophosphate) and 5'-GMP (guanine monophosphate) are important for their flavor-enhancing properties, especially for their synergistic effect with MSG. Nucleic acids for flavor enhancement can be produced in many different ways. RNA is extracted from yeast cell mass and decomposed either chemically or by enzymatic method and then by deamination or phosphorylation to produce IMP, GMP, and AMP. Another method is to produce inosine and guanine from carbohydrate substrate by *Bacillus subtilis* and then phosphorylation to make IMP and GMP. Direct fermentation process involves *Brevibacterium aminogenesis* fermentation on carbohydrate sources. Figure 5.8 shows the different methods of industrial nucleic acid production.

5.4.3 Organic Acids Production

Organic acids are also produced by industrial fermentation process or chemical synthetic methods. Over 70 different organic acids are produced by the fermentation process. Table 5.11 summarizes the microbial strains used for the production of organic acids and their production yield from carbohydrate source.

Enzyme technology is a field aimed at production and improvement of enzymes of interest to the food industry. Modern biotechnology provides the means to select useful enzymes efficiently from conventional fermentation processes.

By using PCR techniques the newly developed enzymes are easily identified. The physiological functions of novel food ingredients are screened and confirmed rapidly by cell culture techniques.

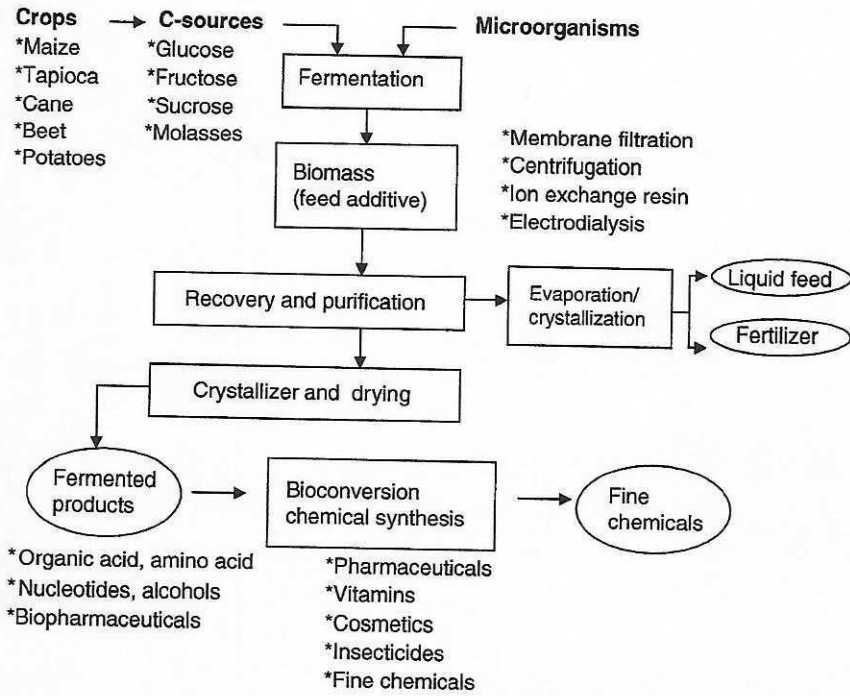


Figure 5.5
Schematic flow diagram of the fermentation bioindustry.

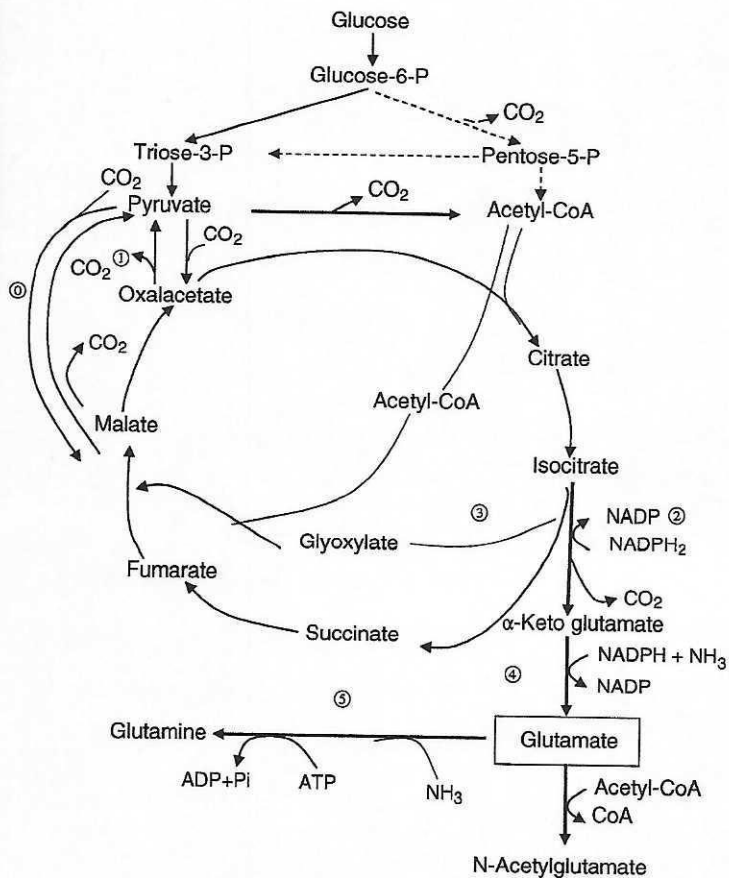


Figure 5.6
Biosynthetic pathways of glutamate production.

Figure 5.7
Flow diagram of the industrial production of glutamate.

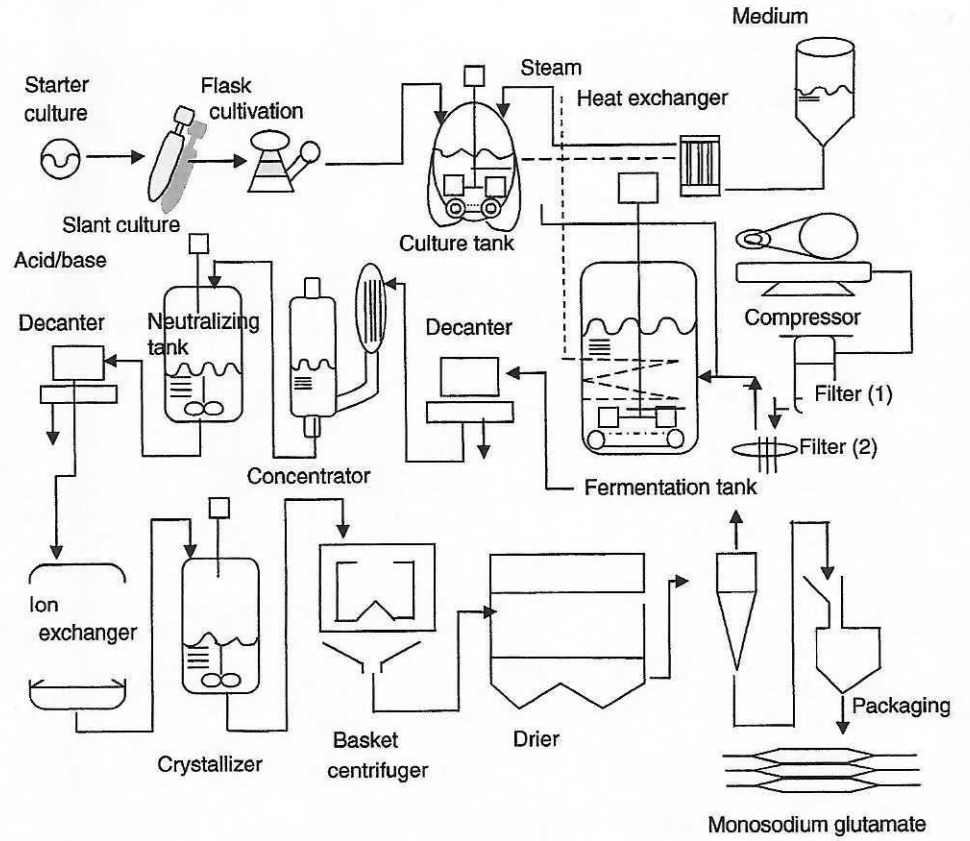
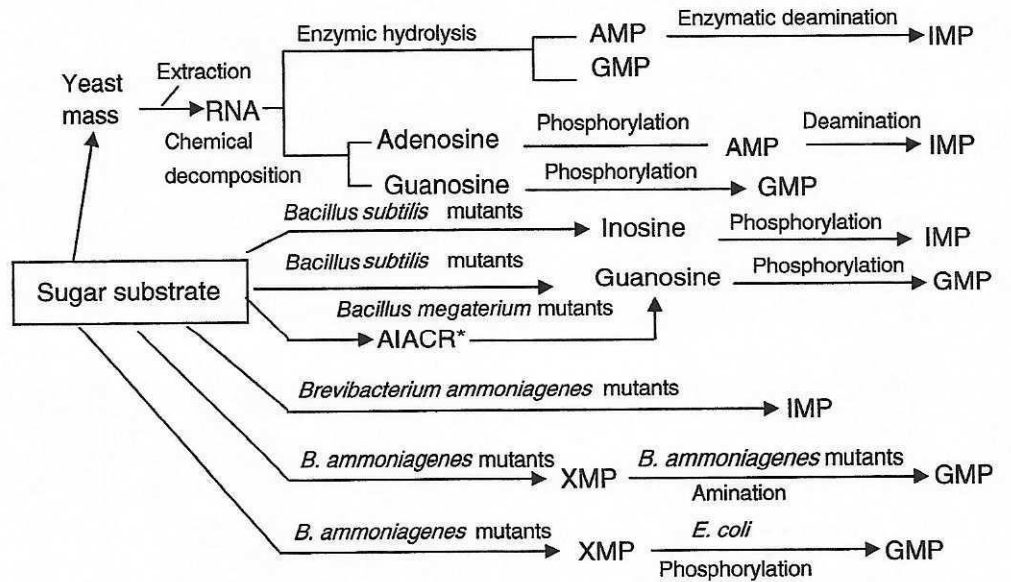


Figure 5.8
Production methods of flavor enhancer nucleic acids.



*5-amino-4-imidazole-carboxamide riboside

Table 5.11

Microbial strains and yields of industrial organic acid production.

Acids	Microorganisms	Yield (%) (C source)
Acetic acid	<i>Acetobacter aceti</i>	95 (Ethanol)
Propionic acid	<i>Propionibacterium shermanii</i>	69 (Glucose)
Pyruvic acid	<i>Pseudomonas aeruginosa</i>	50 (Glucose)
Lactic acid	<i>Lactobacillus delbrueckii</i>	90 (Glucose)
Succinic acid	<i>Cytophaga succinicans</i>	57 (Malic acid)
Tartaric acid	<i>Gluconobacter suboxydans</i>	27 (Glucose)
Fumaric acid	<i>Rhizopus delemar</i>	58 (Glucose)
Malic acid	<i>Lactobacillus brevis</i>	100 (Glucose)
Itaconic acid	<i>Aspergillus terreus</i>	60 (Glucose)
α -Ketoglutaric acid	<i>Candida hydrocarbofumarica</i>	84 (N-paraffin)
Citric acid	<i>Aspergillus niger</i>	85 (Glucose)
L(+)-Isocitric acid	<i>Candida lipolytica</i>	140 (N-paraffin)
L(-)-Alloisocitric acid	<i>Candida brumptii</i>	28 (Glucose)
Gluconic acid	<i>Penicillium purpurogenum</i>	40 (Glucose)
2-Ketogluconic acid	<i>Aspergillus niger</i>	95 (Glucose)
D-Araboascorbic acid	<i>Pseudomonas fluorescens</i>	90 (Glucose)
Kojic acid	<i>Penicillium notatum</i>	45 (Glucose)
	<i>Aspergillus oryzae</i>	50 (Glucose)

5.5 Genetic Engineering

Modern biotechnology is represented by the production of genetically modified organisms and their use in bioindustry. Since GM foods were first introduced in the 1990s, a quiet revolution in the food supply system has been going on. In 2014, 78% of the world's soybean cultivated land and 32% of the world's cornfields were sown with transgenic crops (James, 2014). Most microbial enzymes in use in food production are now produced from GM microorganisms.

5.5.1 DNA Transcription

Each protein is coded for by a piece of deoxyribonucleotide (DNA), commonly known as a gene. In most instances, DNA is located in the chromosome, although in some bacteria important DNA may be found on extrachromosomal elements called plasmids. In plants, mitochondrial and chloroplast DNA as well as nuclear DNA are important. DNA is composed of linear chains of nucleotide bases: adenine pairs with thymine (A-T) and guanine with cytosine (G-C). In DNA double helix, two strands of nucleotides twist round each other, and the strands are linked by bonds between bases in each strand composed of alternating sugar and phosphate sections.

DNA copying is made by breaking the bonds between the bases on each strand and picking up to the free

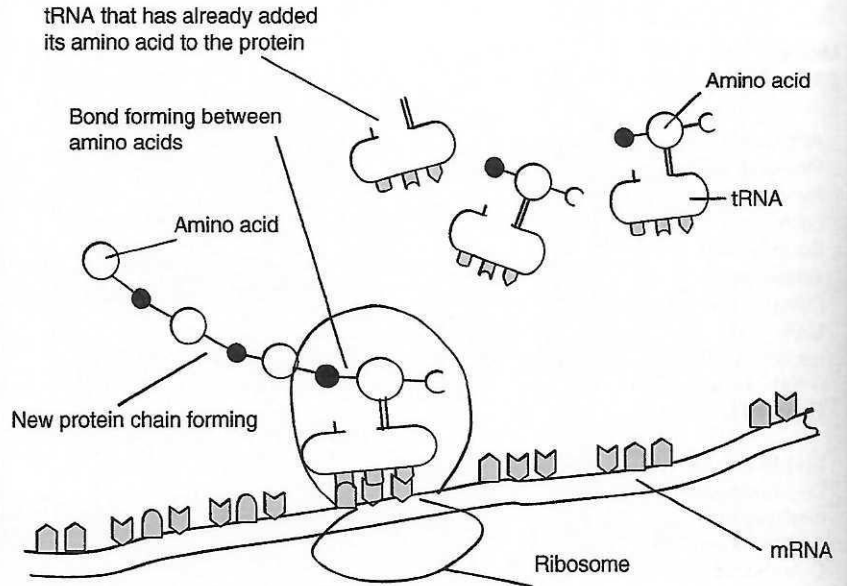
bases new nucleotides from a pool of such molecules provided by the cell. This process occurs with both parent strands, and thus, two identical DNA molecules are created from one original. The specific pairing of the bases guarantees faithful reproduction. The strands of DNA separate and the messenger RNA molecule is built up according to the instructions contained in the DNA strand. As in DNA, the C and G bases pair together, but in mRNA a different base, uracil (U), replaces T as the partner of adenine (A). When the mRNA molecule is complete, it peels off the DNA template and moves to the protein assembly unit 'ribosome.' Ribosomes are built up from several sorts of protein plus a form of RNA called ribosomal RNA. Once the ribosomes have grasped the mRNA, the third type of RNA, transfer RNA (tRNA), comes into action.

There are many sorts of tRNA, and each is able to recognize certain codons on the mRNA. Furthermore, each type of tRNA carries with it just one specific type of amino acid. The translation of genetic code depends on the fact that one end of a tRNA molecule recognizes specific codons, while the other end of the same tRNA molecule carries a particular amino acid. The tRNA deposits the amino acid it has brought on the last amino acid in the growing protein chain. The mRNA then moves another notch along the ribosome, exposing the next codon, and so the process continues. This process is known as transcription and translation.

Figure 5.9 shows mRNA translation for protein synthesis (Prentis, 1984).

Figure 5.9

Schematic diagram of DNA translation for protein synthesis.



5.5.2 Recombinant DNA Technique

Recombinant DNA is made by excising a specific gene from one organism and inserting it into another organism. The development of gene transfer techniques requires a surgically precise means to cut and rejoin pieces of DNA. The discovery of restriction enzymes and ligases, both derived from bacteria in the early 1970s, enabled this. Restriction enzymes are able to cut DNA at specific sites, and ligases are enzymes that rejoin DNA fragments.

Figure 5.10 shows gene cloning in a simple bacterial system (Harlander, 1987). The process of introducing a gene into a host cell is known as transformation. The

proper expressions or functions of transferred genes are tested for conformation. Procedures for transformation and expression in some simple bacteria and unicellular fungi such as *Escherichia coli* and yeast are now well established. Plants and filamentous fungi are more difficult to transform than bacteria or yeast. This is due in part to an increase in the number of chromosomes and the amount of DNA, and to more highly regulated mechanisms of transcription and translation.

Genetic engineering has been applied widely, among others, to crop improvement for high yield, disease resistance, herbicide tolerance, and storage quality enhancement, as shown in Table 5.12.

Figure 5.10

Gene cloning in a simple bacterial system.

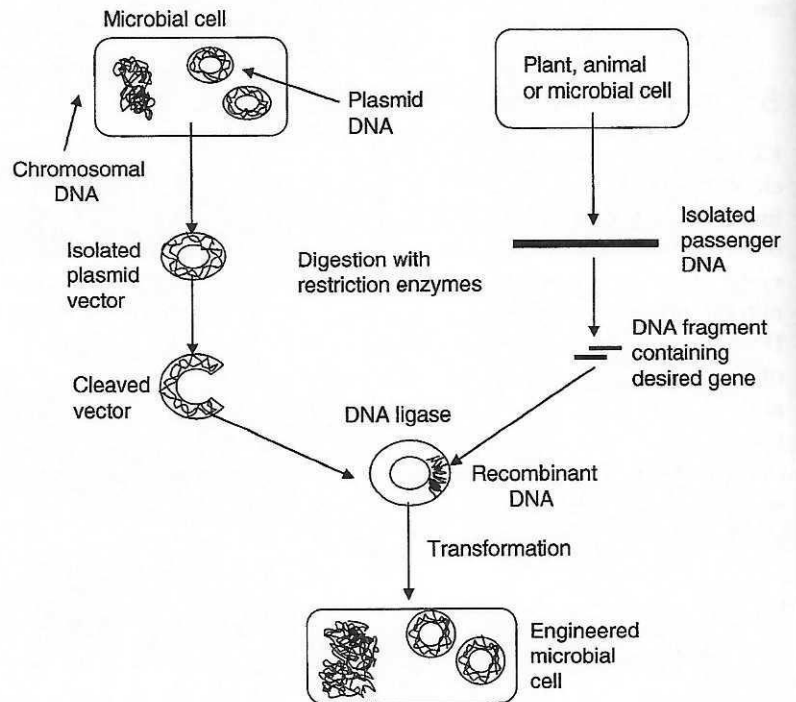


Table 5.12

Application of genetic engineering in the food supply industry.

Agronomic application	Food technology application
Insect protection	Microbial strain improvement for enzyme production
Disease resistance	Ripening modification of fruits and vegetables
Herbicide tolerance	Increased levels of and modification of starch
Virus resistance	Increased levels of and modification of oils
Fungal disease resistance	Improved protein content and quality
Resistance to storage pests	Higher vitamin and mineral contents
Resistance to cold and draught	Reduced cyanogenic glucosides
Nitrogen fixation capability	Improved quality/processing traits

The most immediate applications of genetic engineering in food technology have been in the dairy, baking, and brewing industries. The gene for calf rennin has been isolated and cloned into yeast and fungi to produce calf rennin from microorganisms. The lactose utilization genes of *E. coli* have been cloned into *Sac. cerevisiae*,

Xanthomonas campestris, and other microorganisms so that the lactose in whey permeate can be converted into ethanol, single-cell protein, or xanthan gum. Interspecies gene transfer and protein engineering have been applied to microbial enzyme modification, such as in the production of thermotolerant amylase.

Table 5.13 lists the enzymes produced by GM microorganisms on the market (Robinson, 2001). However, no GM microorganisms have been utilized directly in food production yet.

5.6 Tissue Culture

Plant tissue culture, the propagation of plant tissue in aseptic nutrient media, has widespread application in food and ingredient production (Wasserman *et al.*, 1988). Figure 5.11 shows the tissue culture process (Harlander, 1987). Plant tissue culture plays an important role in plant research, particularly genetic engineering because of time saving. The introduction of new genes into plant tissue requires the generation of single plant cells, or protoplasts. After genetic material is introduced, the protoplasts are grown in tissue culture, and, ultimately, whole plants are regenerated from single cells. Fusion of two genetically dissimilar protoplasts followed by regeneration often results in plants with desirable properties.

Highly valued natural products such as flavors, colors, preservatives, and nutritional supplements are biosynthesized efficiently using this technology. For example, the world's supply of vanilla beans does not satisfy the

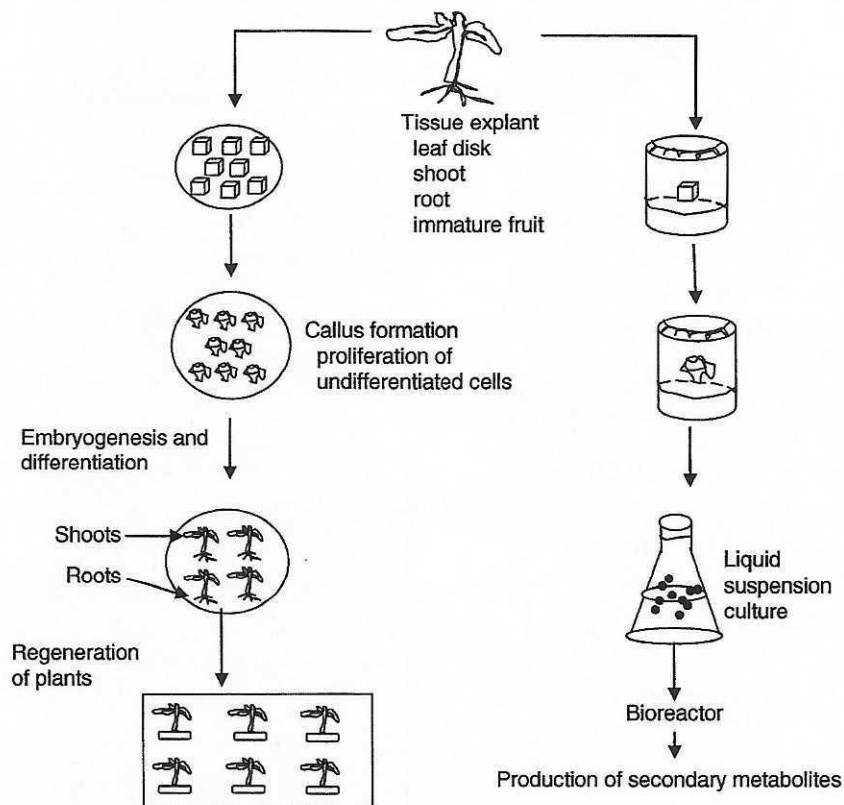
Table 5.13

Examples of commercial food enzymes from GM microorganisms.

Enzyme	Production organism	Food application
Alpha-acetolactate decarboxylase	<i>Bacillus amyloliquefaciens</i> or <i>subtilis</i>	Beverages
Alpha-amylase	<i>Bacillus amyloliquefaciens</i> or <i>subtilis</i>	Baking, beverages
Aminopeptidase	<i>Trichoderma reesei</i> or <i>longibrachiatum</i>	Cheese, dairy
Arabinofuranosidase	<i>Aspergillus niger</i>	Beverages
Beta-glucanase	<i>Bacillus amyloliquefaciens</i> or <i>subtilis</i>	Beverages
Catalase	<i>Aspergillus niger</i>	Egg-based products
Chymosin	<i>Aspergillus niger</i>	Cheese
Cyclodextrin-glucosyl transferase	<i>Bacillus licheniformis</i>	Starch
Glucoamylase	<i>Aspergillus niger</i>	Beverages, baking
Glucose isomerase	<i>Streptomyces lividans</i>	Starch
Glucose oxidase	<i>Aspergillus niger</i>	Baking
Hemicellulase	<i>Bacillus amyloliquefaciens</i> or <i>subtilis</i>	Baking, starch
Lipase, triacylglycerol	<i>Aspergillus oryzae</i>	Fats
Maltogenic amylase	<i>Bacillus amyloliquefaciens</i> or <i>subtilis</i>	Baking, starch
Pectin lyase	<i>Aspergillus niger</i>	Beverages
Pectinesterase	<i>Trichoderma reesei</i> or <i>longibrachiatum</i>	Beverages
Phospholipase A	<i>Trichoderma reesei</i> or <i>longibrachiatum</i>	Baking, fats
Phospholipase B	<i>Trichoderma reesei</i> or <i>longibrachiatum</i>	Baking, starch
Polygalacturonase	<i>Trichoderma reesei</i> or <i>longibrachiatum</i>	Beverages
Protease	<i>Aspergillus oryzae</i>	Cheese
Pullulanase	<i>Bacillus licheniformis</i>	Starch
Xylanase	<i>Aspergillus niger</i>	Baking, beverages

Figure 5.11

Schematic diagram of tissue culture.



demand for vanilla flavor. The *in vitro* biosynthesis of natural vanilla flavor would alleviate this problem (Moshy, 1986). Product yields in plant cell culture are often many-fold higher than those found in the native plant. Technologies used to accomplish this include mutation, adjustment of nutrient and hormone levels, addition of appropriate metabolic precursors, and plant cell immobilization (Knorr and Sinskey, 1985).

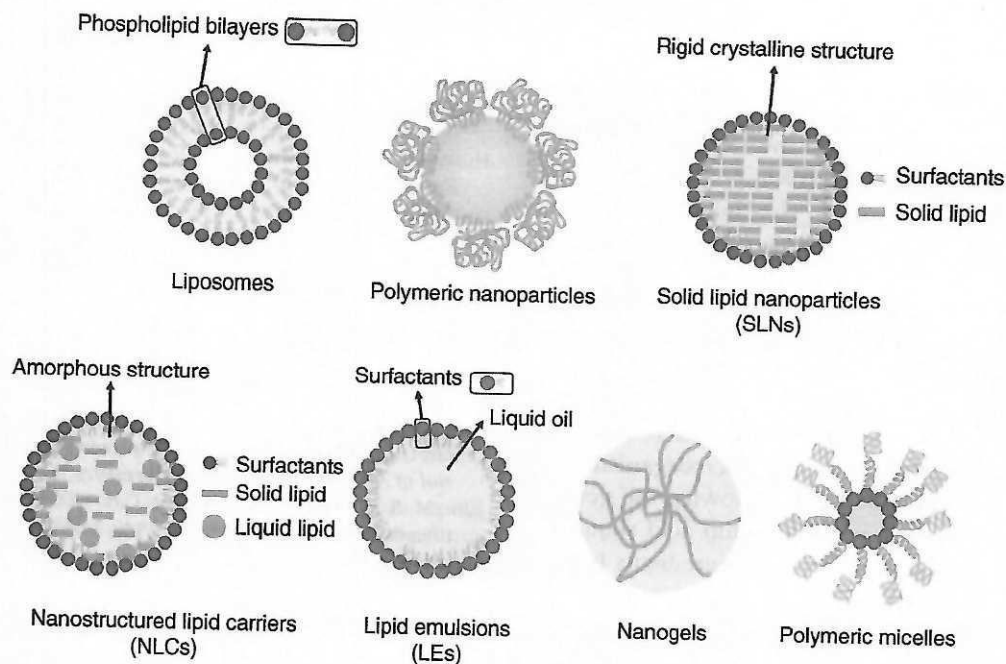
5.7 Nanotechnology in Food Biotechnology

The increasing demands for new types of food as a result of the changes in consumption pattern require development of nanotechnology and its application in food industry. Nanosized-scale manufacturing processes in food may improve the physical, chemical, or biological properties of conventional food or food materials. Nano food material definition is not fixed yet internationally but could be summarized as follows: a food material or a food substance that is manufactured with the average size of nano-scale, or a material or a substance that is composed of 50% or more of the particles (particle numbers) in the nanosized food materials, or a food material or a food substance less than 1 μm (1000 nm) that has similar characteristics as nanosized food mate-

rials that will be different with that of material science in the aspect of size (less than 100 nm) or novel characteristics compared to the same food materials, or an aggregate that is chemically or physically bound with abovementioned materials (FAO/WHO Meeting Report, 2010; Park, 2013).

The nanosized food materials have a much larger surface to mass ratio compared with bulky food equivalents. The nano food-materials enable dispersion of water-insoluble additives (such as colors, flavors, and preservatives) in food products without adding fat or surfactants. Nanosizing of bioactive food materials is claimed to give greater bioavailability in the body compared with bulk food equivalents. Nanosized and nano-encapsulated food ingredients and additives are used for the development of improved or new tastes, flavors and textures, and products with enhanced organoleptic value.

Representative nano delivery vehicles applied in food biotechnology are liposomes, polymeric nanoparticles, lipid nanoparticles, and polymeric micelles (Figure 5.12). Liposomes are spherical vehicles composed of phospholipid bilayer and can encapsulate hydrophilic compounds into their inside and insert hydrophobic compounds into the membrane. Liposomes can be manufactured using sonicators or various homogenizers. Sometimes, liposomes are coated by polymers such as chitosan, which can improve the stability and controlled release profile of liposomes. Polymeric nanoparticles

**Figure 5.12**

Nano delivery vehicles in food biotechnology (adapted and redrawn from Fang, 2012).

are made with polymeric materials, which are two kinds of polymeric nanoparticles such as nanocapsules and nanospheres. Polymeric nanoparticles can be formed by self-assembly of block copolymers consisting of two or more polymer chains with different hydrophobicity. Specifically, the hydrophobic blocks form the core to minimize their exposure to aqueous surroundings, whereas the hydrophilic blocks form a bridge to stabilize the core. The release rates of functional ingredients from the polymeric nanoparticles can be controlled by modifying chemical and physical properties of polymers.

Lipid nanoparticles commonly consist of three different types. Solid lipid nanoparticles (SLNs) are composed of a solid lipid matrix such as wax, which is solid state at room temperature and has a mean diameter of 50 to 1000 nm. The advantages of SLNs are convenience for incorporating both hydrophilic and hydrophobic compounds, without organic solvents. Nanostructured lipid carriers (NLCs) consist of binary mixture of solid lipid such as wax and liquid lipid such as corn oil. NLCs not only possess all of the advantages of SLNs, but also improve the payloads and penetration rate of compounds due to their lower fluidity, which can improve the bioavailability. Lipid emulsion (LE) systems are commonly O/W emulsions that are composed of surfactants. There are two types of LEs, microemulsion and submicron lipid emulsion. The mean size of microemulsion is less than 150 nm, and mean diameter of submicron lipid emulsion

ranges from 100 to 500 nm. Microemulsions usually need a high surfactant concentration (>20%, w/w), but submicron lipid emulsions can be manufactured with 5–10% (w/w) surfactant concentration.

Nanogels are hydrogel state formed by chemically or physically cross-linked polymer networks of nanoscale size, ranging in size from 100 to 500 nm. Nanogels can facilitate the encapsulation of various bioactive compounds. Nanogels usually respond to different environmental conditions that will give controlled release property.

Polymeric micelles are nanosized core/shell structures formed by amphiphilic block copolymers (Croy, 2006). Above critical micelle concentration, these polymers form spontaneously polymeric micelles in a given specific condition. Polymeric micelles have a covalent linkage between individual surfactant molecules (Mourya, 2011). There are big differences in solubility between hydrophilic and hydrophobic segments, known to assemble in an aqueous medium into polymeric micelles. These micelles have a low polydispersity index and are characterized by their hierarchical core-shell structure.

Examples of nanotechnology use in food biotechnology under investigation are vitamin C loaded nanoliposomes (Liu and Park, 2010), chitosan-coated curcumin nanoliposomes (Shin *et al.*, 2013), and capsaicin nanoemulsions (Kim *et al.*, 2014).

5.8 Future Prospects

The biotechnological revolution we have witnessed in the last century has opened up the possibility of processing food through specific reaction and producing food having specific function. The initial application of genetic engineering for agronomic benefits, such as resistance to herbicides and plant diseases, shorter time between sowing and harvesting, and increased yields, is now gradually changing focus to quality traits for producing better nutritional value and maintaining quality. Although the direct use of GM microorganisms in food has not yet occurred in the marketplace, new and powerful enzymes derived from GM microorganisms are widely used in food processing. By knowing the genetic map of major food crops and the amino acid sequence of proteins, the tailor-making of food ingredients having specific function is now possible through biotransformation and biocatalysis (Lee, 2003).

Modern biotechnology provides the means to select useful enzymes efficiently from conventional fermentation processes. By using the PCR technique, the newly developed enzymes are easily identified. The physiological functions of novel food ingredients are screened and confirmed rapidly by cell culture technique. The bioavailability and functionality of biotechnology products are enhanced by applying newly emerging nanotechnology in food processing.

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