

**SOLID SUBSTRATE FERMENTATIONS, ENZYME PRODUCTION, FOOD ENRICHMENT**

**YUSUF CHISTI**  
University of Almería  
Almería, Spain

**KEY WORDS**

Bioreactors  
Enzymes  
Fermentors  
Fermented foods  
Indigenous fermented foods  
Leaching  
pH control  
Product recovery  
Scale-up  
Temperature control  
Water activity

**OUTLINE**

Introduction  
Fermentation Equipment

Chisti, Y., in *Encyclopedia of Bioprocess Technology: Fermentation, Biocatalysis, and Bioseparation*, vol. 5, Flickinger, M. C. and Drew, S. W., editors, Wiley, New York, 1999, pp. 2446–2462. Solid substrate fermentations, enzyme production, food enrichment.

- Tray Fermentors
- Static Bed and Tunnel Fermentors
- Rotary Disk Fermentors
- Rotary Drum Fermentors
- Fluidized Beds
- Agitated Tank Fermentors
- Continuous Screw Fermentors
- Other Systems
- Construction and Cleaning
- Design and Scale-Up
- Environmental Factors in Solid Substrate Fermentations
  - Moisture
  - Temperature
  - pH
- Production of Enzymes
  - Process Technology
  - Regulatory Considerations
- Other Products and Applications
  - Secondary Metabolites
  - Environmental Remediation
  - Animal Feeds
- Product Recovery from Fermented Solids
- Fermentation-Enriched and Fermentation-Modified Foods
  - Miso
  - Sake
  - Soy Sauce
  - Tempe
  - Oncom
  - Sausages
  - Coffee
  - Cocoa
  - Mushrooms
- Safety Considerations
- Bibliography

## INTRODUCTION

Solid substrate fermentations involve microbial modification of a solid, undissolved substrate. This definition includes solid-state fermentations in which microbial cultures grow on a moist solid with little or no free water, although capillary water may be present (1). Examples of this type are seen in mushroom cultivation (2-4), bread making (5,6), and production of cheeses (7). Examples in which the solid substrate is submerged in large amounts of free water are pickling of vegetables (8) and brewing of soy sauce and sake (9,10), as well as bioconversion of cellulosic materials (11,12).

Solid substrate fermentations have been practiced worldwide since ancient times (9,13,14). Bread, sausages, and soy sauce are a few familiar products of solid substrate fermentation. Such fermentations are used in processing

coffee and cocoa, as well as in environmentally significant operations: composting, silage production, soil bioremediation, biodesulfurization of coal, and metal recovery from low-grade ores (15). Solid substrate fermentations are employed also in producing enzymes and chemicals and in biotransformations.

Despite long history and widespread use, most solid substrate fermentations are not well understood. Processing is often an art, commonly employing poorly defined, naturally occurring, mixed microbial populations. Mixed cultures are sometimes essential to development of the desired product characteristics, including appearance, aroma, texture, and taste. With few exceptions, fermentations are labor-intensive batch operations. The level of technical sophistication and capital outlay vary a great deal. Solids concentration is generally high, whereas the water content is usually low (16). The product may be highly concentrated in comparison with submerged fermentations. Similarly, water consumption is comparatively lower, but space requirements can be substantial. Solid substrate culture generally requires less power because agitation is not excessive. A low-pressure blower is sufficient for air supply, unlike the higher-pressure compressors required in submerged fermentations (17). Unlike submerged culture, solid-state processes require extensive solids handling, which is difficult; nevertheless, highly mechanized, automated, and continuous processing of solids is being practiced in large *koji* (or molded grain) factories (9,10,18), as well as for other processes. Processing machinery tends to be complex. Hygienic processing practices are followed, but sterility standards that are common in submerged culture production of pharmaceuticals are not attained. Because of fungal spores and product- or microbe-contaminated dust from substrate solids, solid substrate fermentations often pose a greater health risk to operators than do submerged fermentations.

## FERMENTATION EQUIPMENT

Solid substrate fermentation devices vary in technical sophistication from the very primitive banana leaf wrappings to highly automated machines used mainly in Japan. Simple fermentation systems (e.g., fermentation of cocoa beans in heaps) are quite effective in some large-scale processes. Commonly used fermentation devices are detailed in the following sections.

### Tray Fermentors

One of the simplest and widely used fermentors is a wooden, metal, or plastic tray, often with a perforated or wire mesh bottom to improve air circulation (1,19,20). A shallow layer, usually less than 0.15 m deep, of pretreated (e.g., steamed) substrate is placed on the tray for fermentation. Individual trays or stacks may be located in temperature- and humidity-controlled chambers (Fig. 1) or simply in ventilated areas. A spacing of at least one tray height is usually allowed between stacked trays. Trays may be covered with cheesecloth to reduce contamination (10), but strict monosepticity is not attempted. Inoculation and occasional mixing are done manually, often by hand

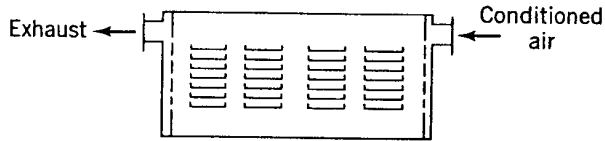


Figure 1. Tray fermentation chamber.

(10). This type of technology is quite common in small- and medium-scale koji operations in Asia. In some cases, filling, emptying, and other aspects of handling (4) of trays may be automated. For example, hinged trays with open ends may be dropped for emptying (19). Despite some automation, tray fermentors are labor intensive, require a large area, and difficulties with processing hundreds of trays limit their scalability (19). In parts of Asia, the bamboo baskets historically used instead of trays (16) continue to be employed.

**Static Bed and Tunnel Fermentors**

A commercially used modification of the tray fermentor employs a single, larger and deeper, static bed of substrate with forced aeration through the bed (Figs. 2 and 3). The substrate is located in an insulated chamber. In one version of this device, the tunnel fermentor (Fig. 3), the bed of solids may be quite long (1,20), but is usually no deeper than 0.5 m. Tunnel fermentors may be highly automated with mechanisms for mixing (Fig. 3), inoculation, continuous feeding, and harvest of substrate.

**Rotary Disk Fermentors**

Some large-scale commercial koji-making operations in Japan use a rotary disk fermenter configuration shown schematically in Figure 4 (18). The fermentor consists of upper and lower chambers, each with a circular perforated disk

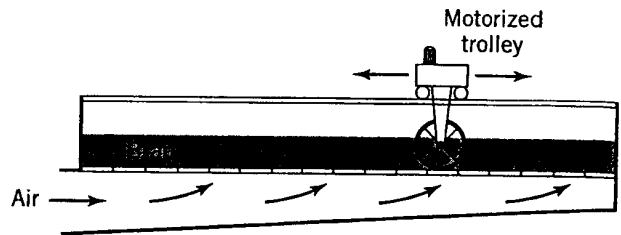


Figure 3. Mechanical mixing of fermenting substrate in a tunnel fermentor.

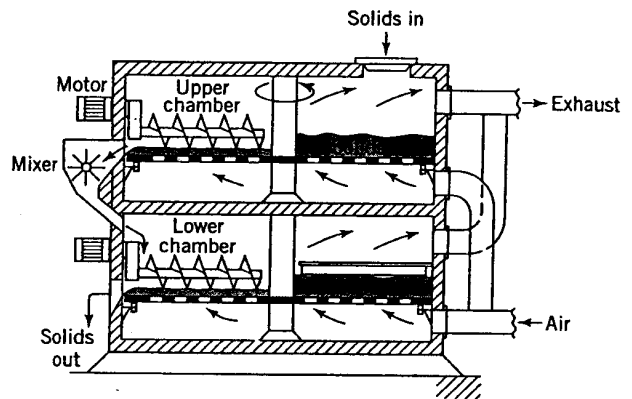


Figure 4. Automatic rotary koji fermentor.

to support the substrate. A common central shaft rotates the disks. Inoculated substrate is introduced in the upper chamber and slowly moved to the transfer screw. The upper screw transfers the partly fermented solids through a mixer to the lower chamber where further fermentation occurs. The mixer breaks up the partly fermented

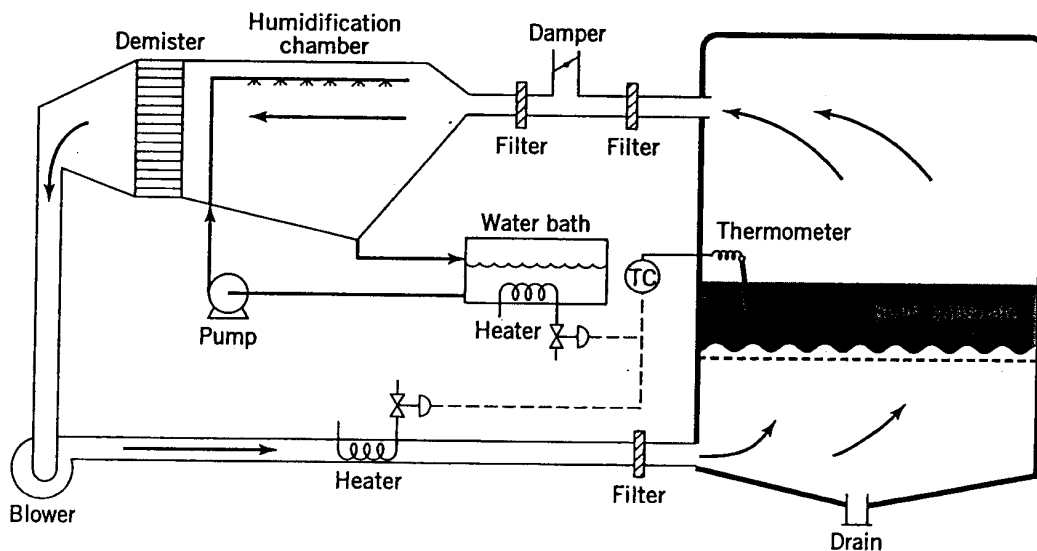


Figure 2. Static bed-type koji fermentor.

substrate-mycelium aggregates halfway through the fermentation process. Fermented substrate is eventually harvested using the lower transfer screw (Fig. 4). Both chambers are aerated with humidified, temperature-controlled air.

A tower fermentor similar in concept to the rotary koji fermentor is used in certain composting operations. The solids are supported on plates or trays that may be perforated. A stack of several tray chambers forms the tower. Mechanical devices mix and move solids down the tower (20–22) in a manner similar to that described for the rotary koji device. The gradually moving bed of solids is aerated either by forced aeration through the solid mass or solely from the surface exposed to the air above the bed.

### Rotary Drum Fermentors

Rotary drum fermentors consist of a cylindrical vessel mounted horizontally on rollers and rotated around the long axis (Fig. 5). The rotational speed is commonly 1–5 rpm, but speeds up to 15 rpm are used occasionally. Rotation may be intermittent, and the speed may vary with the fermentation stage. The vessel may have straight or curved internal baffles that aid aeration and temperature control by imparting a tumbling motion to the substrate (23). Sometimes the drum may be inclined, causing the substrate to move from the higher inlet end to the lower outlet during rotation. The amount of substrate that can be processed in a batch operation depends directly on the length of the vessel and the square of its diameter. Aeration is through coaxial inlet and exhaust nozzles (Fig. 5). The air inlet pipe may extend into the vessel and may branch into several arms (19). Air is either supplied by a compressor or may be sucked in by an exhaust fan located at the air outlet (19).

### Fluidized Beds

Fluidized-bed fermentors (Fig. 6) are relatively uncommon, but sufficiently promising that large-scale devices are being tested (9). A fluidized-bed fermentor consists of a relatively shallow bed of substrate supported on a perforated plate. Compressed conditioned air is normally used for fluidization, but carbon dioxide or nitrogen may be employed for anaerobic processes. Superficial gas velocity for fluidization is typically  $0.24\text{--}1\text{ m s}^{-1}$ ; the specific value

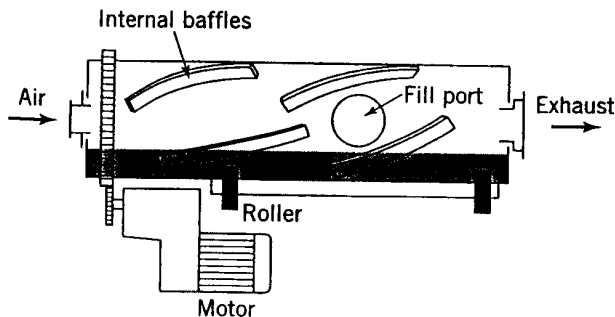


Figure 5. Rotary drum fermentor.

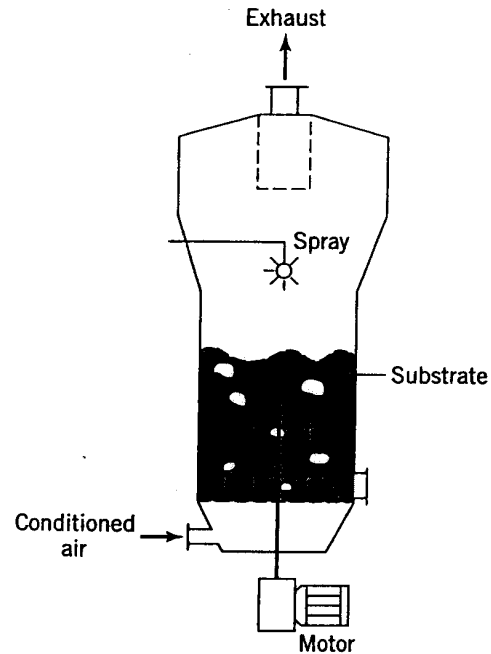


Figure 6. Fluidized-bed fermentor.

depends on the density and the particle size of the substrate. In the upper regions of the fermentor, the cross section is expanded (Fig. 6) to reduce the gas velocity to values that are insufficient to suspend the larger particles. Loss of fines in the exhaust gas is prevented by screens, filters, or cyclonic separators located at the exhaust nozzle. Fluidized beds achieve good aeration, mixing, and temperature control. Intermittent water spray may be necessary to prevent dehydration. A mechanical agitator as in Figure 6 is useful for periodic loosening of clumped solids.

### Agitated Tank Fermentors

Helical ribbon-stirred tank fermentors have been employed for solid-state culture of fungi such as *Chaetomium cellulolyticum* on wheat straw (24). Other similar designs have utilized multiple helical screws for agitation of large rectangular tanks. The screws extend into tanks from mobile trolleys that ride horizontal rails located above the tanks (25). Yet another stirred tank configuration is the paddle fermentor. This design is similar to the rotary drum device (Fig. 5), except that the drum is stationary and motor-driven paddles on a concentric shaft provide periodic mixing of the substrate (20). As with rotary drums, the quantity of the substrate in paddle fermentors is generally restricted to less than 50% of the volume of the vessel. Although simpler than the rotary drum, the paddle fermentor is unlikely to be as effective in moving the entire mass of solids.

### Continuous Screw Fermentors

A screw fermentor suitable for continuous fermentations is shown in Figure 7. Sterilized, cooled, and inoculated sub-

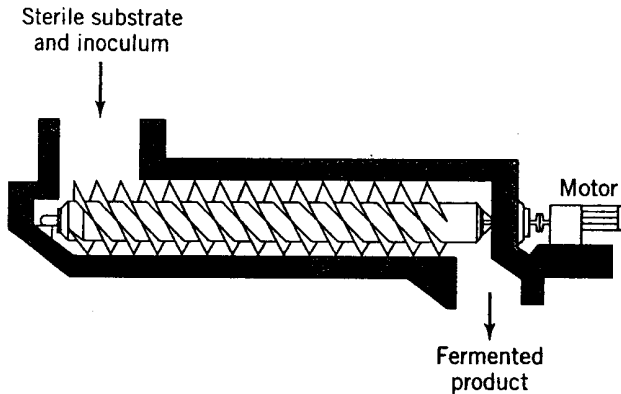


Figure 7. Screw fermentor for continuous fermentation.

strate is fed at the inlet. The screw moves the fermenting solids toward the harvest port. The fermentation time depends on the rotational speed and the length of the screw. Because the device is not aerated, only anaerobic or microaerophilic fermentations may be carried out.

#### Other Systems

Other miscellaneous types of fermentation devices that are used mostly in small-scale studies have been described by Lonsane et al. (19). Equipment for fermenting undissolved solid substrate slurries is identical to that used in submerged culture processes. Exceptions may be in devices used to feed slurried solids to the fermentor and remove the fermented product. Commonly used slurry fermentors include stirred tanks, bubble columns, and air-lift vessels (26–28). Simple vats are frequently employed in anaerobic slurry fermentations. Fermentors used in various types of composting have been described (14,21,22).

#### Construction and Cleaning

Modern commercial solid-state fermentors are constructed predominantly of stainless steel, but pressure vessel construction that is the norm for submerged culture processes (29) is not employed. Large concrete or brick fermentation chambers, or koji rooms (Fig. 1), may be lined with steel. Type 304 steel is used typically, but more corrosion-resistant grades such as Type 304L and 316L are also utilized. Materials of construction have been discussed further by Chisti (29).

Because pressure vessel construction is not used, the fermentors cannot be steam sterilized; however, the design usually allows for atmospheric steaming. Typically, the fermentation chamber and process vessels are washed with alkaline detergent, sanitized with sodium hypochlorite, formaldehyde, or quaternary ammonium salts, and steamed between successive batches (17). Automated clean-in-place methods similar to those noted for submerged culture processes (30) are increasingly employed.

#### Design and Scale-Up

Design and scale-up of solid-state fermentation equipment relies heavily on empirical experience because the process

kinetics and transport phenomena, such as heat transfer, oxygen transfer, and mixing, in these systems are insufficiently understood for more formal approaches to be fully applied (20). Potentially useful strategies for scale-up have been discussed by Lonsane et al. (31), and Moo-Young et al. (22) have considered aspects of transport phenomena.

### ENVIRONMENTAL FACTORS IN SOLID SUBSTRATE FERMENTATIONS

#### Moisture

Moisture content of the substrate is an important consideration in solid substrate processes. Free water indicates a saturated substrate. The moisture content at which free water is apparent depends on the nature of the solid (Table 1). Many solid substrate fermentations are carried out with little or no free water; typically, the substrate moisture content is 30–80% w/w (1,32). High moisture levels lead to aggregation of substrate particles, poor aeration, and possible anaerobic conditions (24). Steamed rice, a common substrate, becomes sticky when the moisture level exceeds 30–35% w/w. Optimal moisture content depends on the microorganism and the substrate. For the commonly used fungi, the optimal moisture requirement varies between 40% and 80% w/w. For the same organism growing on different substrates, the optimal moisture levels may differ widely; hence, percent moisture by itself is unreliable for predicting growth (32), and water activity is preferred instead. The water activity or relative humidity of the substrate is the ratio of the vapor pressure of water in the substrate to the vapor pressure of pure water at the temperature of the substrate. Water activities below 0.9 do not support most bacterial growth, but yeasts and fungi can grow at water activities of 0.7 and greater. Thus, the low moisture environment of many solid substrate fermentations favors yeasts and fungi. The water activity in a substrate may be determined by hygrometric measurement of relative humidity of a small air space in equilibrium with the substrate.

In addition to affecting growth, water activity affects product formation. The spectrum of products produced and characteristics such as aroma are affected. In some cases, the optimal water activities for growth and product formation differ (32). The optimal water activity depends also on factors such as agitation rate and cultivation temperature (32). Because the water activity depends on the concentration of dissolved solutes, sometimes salts, sugar, or other solutes are added to alter the activity. Different additives may influence the fermentation differently even though the water activity produced may be the same. Furthermore, the fermentation process itself leads to changes

Table 1. Saturation Moisture Content of Some Substrates

Substrate	Percent moisture (w/w)
Maple bark	>40
Rice and cassava	>50–55
Straw	75

in water activity as products are formed and the substrate is hydrolyzed. Oxidation of carbohydrates produces water.

During fermentation the water activity is controlled by aeration with humidified air and, sometimes, by intermittent water spray. Aeration with water-saturated air has commonly been found to increase the moisture content of the substrate. Relative humidity of the aeration gas is typically 60–80%. Ideally, prevention of water loss or gain by the uninoculated substrate would require that the water activity of the aeration gas and the substrate be identical. In practice, water generated during fermentation, and the additional requirement of evaporative cooling, demand aeration gas that is somewhat drier than the substrate. The temperature and humidity of the aeration gas are controlled in the same way as in air conditioning systems: temperature and humidity sensors (hygrometers) in the fermentation chamber provide the necessary data, and cooler air is humidified by water spray or injection of clean steam, and warmed to reduce the humidity to the required value (Fig. 2). Humidity–temperature relationships available in standard psychrometric charts are the basis of control.

### Temperature

At 10–30 kg m<sup>-3</sup>, the maximum microbial biomass concentration in solid-state fermentations is lower than 40–50 kg m<sup>-3</sup> typically seen in submerged culture (24); but, because there is little water, the heat generation per unit fermenting mass tends to be much greater in solid-state fermentations. Temperature can rise rapidly, again, because there is little water to absorb the heat (i.e., the mean specific heat capacity of the fermenting mass is much lower than that of water). Consequently, temperature control in large-scale fermentations can be particularly difficult. Removal of the metabolic heat sometimes becomes the major limitation, especially because of the poor thermal conductivity of the porous fermenting mass. Cumulative metabolic heat generation in koji fermentations for a variety of products has been noted at 419–2,387 kJ kg<sup>-1</sup> solids (14). Higher values, up to 13,398 kJ kg<sup>-1</sup>, have been observed during composting (32). Peak heat generation rates in koji processes range over 71–159 kJ kg<sup>-1</sup> h<sup>-1</sup>, but the average rates are more moderate, at 25–67 kJ kg<sup>-1</sup> h<sup>-1</sup> (32). Peak metabolic heat production rate during fermentation of readily oxidized substrates such as starch can be much greater than in typical koji processes. For example, in one case, a generation rate of 330 kJ kg<sup>-1</sup> h<sup>-1</sup> was observed for *Aspergillus niger* growing on cassava starch. Heat generation during composting has been treated in depth by Stentiford and Dodds (33).

Temperature control during fermentation is obtained mostly through evaporative cooling (34); hence, drier air provides a better cooling effect. Air temperature is controlled. Intermittent spray of cool water is sometimes necessary to prevent dehydration of the substrate. Occasionally, the substrate-containing metal trays are also cooled by a circulating coolant, even though most relatively dry and porous substrates are poor conductors. Intermittent agitation further aids heat removal, particularly in large heaps and piles. Despite much effort, temperature gradi-

ents in the substrate do occur, particularly during peak growth. Gradients as steep as 3 °C cm<sup>-1</sup> have been recorded during rapid growth of *Rhizopus oligosporus* on soybeans in *tempe* fermentations, even though the substrate layer was less than 7 cm deep (35). Similar values were noted in solid-state citric acid production by *Aspergillus niger* (34).

### pH

Solid-state fermentations are practiced without pH control (19) other than any adjustments made during substrate preparation. In some nonsterile processes the initial pH may be adjusted to pH 4 or less to suppress bacterial contamination. Unlike most bacteria, yeasts and fungi are generally tolerant of more acidic conditions. Either mineral acids (e.g., 0.2 M hydrochloric acid) or organic acids (e.g., 0.5% w/w acetic acid) may be used for pH adjustments. Other than initial adjustments, the buffering capacity of substrates is relied on to check large changes in pH during fermentation (19). Many substrates are effective buffers. This is particularly true of protein-rich substrates, especially if deamination of protein is minimal. Some pH stability can be obtained by using a combination of urea and ammonium sulfate as the nitrogen source in the substrate. Decomposition of urea produces ammonia that takes up a proton to become NH<sub>4</sub><sup>+</sup>, causing an increase in pH. This effect is countered by ejection of a proton from cells that take up the ammonium ion but incorporate it into proteins as —NH<sub>3</sub><sup>+</sup>. Uptake of ammonium causes rapid acidification. In the absence of other contributing nitrogen sources, an equimolar combination of ammonium sulfate and urea is expected to yield the greatest pH stability. In media that are rich in protein or amino acids, deamination may contribute to pH rise. Attention to stabilizing the pH during fermentation can be particularly important for certain processes. Stability of enzymes and some secondary metabolites is pH dependent, and even though the rate of production may not be affected by changes in pH, the overall process productivity may decline because of destruction of the product. This behavior has been observed in producing pectinases by *Aspergillus niger* cultured on glucose-enriched sugar cane bagasse (36).

## PRODUCTION OF ENZYMES

### Process Technology

Only extracellular enzymes, those secreted by the microbial cell into the extracellular medium, can be produced by solid-state fermentation technology (37). Predominantly, these are hydrolytic enzymes (38,39), as noted in Table 2. Commercial production of many of those enzymes in the West utilizes submerged culture, but solid-state fermentation persists particularly in Asia where koji-type processing is commonly used. Koji is molded grain that has been used in oriental food preparations for thousands of years (40,41). Koji is a source of fungal enzymes that digest proteins, carbohydrates, and lipids into nutrients used by other microorganisms in subsequent fermentation. Koji

Table 2. Enzymes That May Be Produced by Solid-State Fermentation

Enzyme	Producing organisms
Amylase	<i>Aspergillus oryzae</i> , <i>Aspergillus niger</i> , <i>Bacillus licheniformis</i> , <i>Bacillus subtilis</i> , <i>Bacillus megaterium</i>
Catalase	<i>A. oryzae</i> , <i>Rhizopus niveus</i>
Cellulase	<i>Trichoderma reesei</i> , <i>Trichoderma viride</i> , <i>Trichoderma koningi</i> , <i>Trichoderma harzianum</i> , <i>Aspergillus ustus</i> , <i>A. oryzae</i> , <i>Sporotrichum pulverulentum</i> , <i>Penicillium spinulosum</i> , <i>Penicillium capsulatum</i> , <i>Pestalotiopsis versicolor</i>
Chitinase	<i>A. niger</i>
Glucoamylase	<i>A. niger</i> , <i>A. oryzae</i> , <i>Rhizopus</i> sp.
Invertase	<i>Aspergillus awamori</i> , <i>A. niger</i> , <i>A. oryzae</i>
Lactase	<i>A. oryzae</i>
Linamarase	<i>Aspergillus sydowi</i> , <i>Penicillium steckii</i>
Lipase	<i>A. niger</i> , <i>Aspergillus luchuensis</i> , <i>Rhizopus delemar</i> , <i>Penicillium candidum</i>
Pectinase	<i>A. niger</i> , <i>Aspergillus sojae</i> , <i>Aspergillus fumigatus</i> , <i>Bassochlumys fulva</i> , <i>Penicillium expansum</i>
Phytase	<i>Aspergillus ficuum</i> , <i>Rhizopus oligosporus</i>
Protease	<i>A. oryzae</i> , <i>A. niger</i> , <i>Aspergillus flavus</i> , <i>Mucor dispersus</i>
Rennet	<i>Mucor pusillus</i> , <i>Mucor meihei</i> , <i>R. oligosporus</i>
Ribonuclease	<i>Aspergillus candidus</i>
Xylanase	<i>A. niger</i> , <i>A. fumigatus</i> , <i>Aspergillus terreus</i> , <i>Gibberella fujikuroi</i>

comes in many varieties depending on the mold, substrate, method of preparation, and stage of harvest. Traditional and mechanized koji production has been described by several sources (9,10,18), and concise outlines of food koji processes are given later in this article. In typical processing for enzymes, a suitable solid substrate is mixed with water and mineral salts, heat sterilized/cooked, cooled, and inoculated. Incubation at controlled temperature (20–45 °C) and humidity follows. Subsequently, the fermented substrate is extracted with buffer to yield an enzyme-containing liquor. Further purification and recovery methods are identical to those that have been described for submerged culture processes (17,27,42,43). The crude liquor is filtered or centrifuged to remove suspended solids and concentrated by ultrafiltration. The concentrate may be stabilized and packaged. Extensive purification is uncommon for many bulk enzymes. In some cases, the fermented substrate may be dried at 35–40 °C, and ground before extraction (44).

The commonly used solid substrates include wheat bran, wheat, soybean, rice, barley, oats, and other cereals, but many more substrates have been used occasionally (Table 3). Studies with nutritionally inert substrates (e.g., polyurethane foam) wetted with dissolved nutrients have been reported (45). Two or more substrates are sometimes used in combination. Cereal grain may be used whole or cracked into pieces. Substrate particle size affects the extent and the rate of microbial colonization, air penetration, and carbon dioxide removal as well as the downstream extraction and handling characteristics (19,46). Relatively small particles are preferred because they present a larger surface for microbial action. However, particles that are too small pack together to reduce the interparticle voids that are essential for aeration (46). Similarly, too many fines in a batch of larger particles will fill up the voids. Particle shapes that pack together tightly (e.g., flat flakes, cubes) are undesirable. Lignocellulosic substrates such as straw, sugar cane bagasse, and wood chips are sometimes extensively pretreated to ease microbial colonization and substrate utilization. Pretreatments include size reduction, steaming, steam explosion, solubilization of lignin with or-

Table 3. Solid-State Fermentation Substrates

Animal	Milk solids
Apple pulp	Millet
Bagasse	Newspaper
Banana meal	Nutrient wetted inerts
Barley	Oats
Beet pulp	Orange peel
Buckwheat	Paper pulp
Canola meal	Peanut press cake
Cardboard	Potato starch
Cassava starch	Rice
Coffee pulp	Rice bran
Corn cobs	Rye meal
Corn grits	Sawdust
Cotton seed meal	Sewage sludge
Fish	Soybean
Fruit pulp	Straw
Jerusalem artichoke meal	Sweet potato meal
Lignocellulosic waste	Wheat bran
Logs	Wood chips
Meat	Vegetables

ganic solvents, acid and alkaline hydrolysis, and irradiation (12,47,48). Most of those pretreatments have proven too expensive for commercial utilization. For cereal substrates, common pretreatments include dehulling, pearling, size reduction, soaking, and cooking or steaming (9,10,18).

The selection of substrate depends to some extent on the enzyme of interest. Thus, pectinases are often produced using fungi grown on fruit pulp (e.g., apple, avocado), or on a combination of pulp and bran, or bran and fruit juice. Similarly, cellulose-rich substrates such as rice and wheat straw are commonly employed in producing cellulases.

Enzyme production processes rely overwhelmingly on filamentous microfungi (43), the main producer genera being *Aspergillus*, *Mucor*, *Penicillium*, *Trichoderma*, and *Rhizopus*. *Bacillus* species predominate among bacteria used in enzyme production (38,43,44). Examples of pro-

duction from yeasts are few. Some specific enzyme producers are listed in Table 2. Production of  $\beta$ -galactosidase by the yeast *Kluveromyces lactis* grown on corn grits and wheat bran moistened with deproteinated milk whey has been reported (49). Although  $\beta$ -galactosidase is an intracellular enzyme in yeasts and cultivation on solid substrate does not promote secretion, solid-state fermentation has been claimed to be superior to liquid culture for this enzyme (49); however, recovery questions have not been addressed.

Microbial inocula for commercial enzyme production are maintained as pure cultures (17). Routine checks on culture purity, freedom from variant forms, physiological characteristics, and enzyme productivity are used to ensure process consistency (17). The sterile solid substrate is inoculated in one of several ways: by mixing with a liquid suspension culture inoculum; by mixing with solid substrate-grown inoculum; by using a suspension of solid substrate-produced spores in a sterile liquid; or by blowing the solid substrate-produced spores with sterile air going into the fermentation chamber. Aseptic processing practice is observed (17), but fermentations are never entirely contamination free. Fermentations last 1–7 days, and a significant amount of the substrate is oxidized to carbon dioxide and water (17). Thin uncompacted and porous layers of nonadhering solid substrate particles generally ensure good oxygen penetration from the exposed surface. Excessive agitation is not wanted. Occasional turning and mixing improve oxygen transfer and reduce compaction and mycelial binding of substrate particles. Because agitation continually damages the surface hyphae, mixing suppresses often-unwanted sporulation (46). The frequency of agitation may be purely experience based, as in occasional turning of a fermenting cocoa heap, or it may be determined by a temperature controller.

Production of spore inocula requires static conditions. Deep substrate layers and heaps may require forced aeration and agitation. Oxygen supply affects product formation. Depending on the microorganism and the product, both enhanced and reduced productivities have been observed to accompany improved aeration or agitation. Aeration plays an important role in removing carbon dioxide and in controlling temperature and moisture. In some cases, increased concentrations of carbon dioxide have been severely inhibitory, whereas enhanced oxygen partial pressures have improved productivity. If used, forced aeration rates may vary widely; a typical range being  $0.05\text{--}0.2 \times 10^{-3} \text{ m}^3 \text{ kg}^{-1} \text{ min}^{-1}$  (1).

### Regulatory Considerations

Production of food, medicinal, and medical–diagnostic enzymes must comply with standards of “good manufacturing practices” as established by the U.S. Food and Drug Administration (FDA) or other regulatory agency appropriate to the particular jurisdiction. Safety of enzyme preparations, especially those derived from non-GRAS species (GRAS, generally recognized as safe; i.e., microorganisms such as baker’s yeast that have proven to be safe through extended use and have been recognized as such by placement on the GRAS list of the FDA), must be demonstrated

to gain marketing approval. Specific requirements vary with the intended use of the product. Freedom from toxins, antibiotics, other contaminants, and unwanted effects may have to be ascertained. Multigeneration feeding trials and studies of teratogenicity may be necessary. Requisite purity, potency, and stability may have to be demonstrated. The quality assurance requirements are similar to those for therapeutic proteins produced by other methods (42,50–52). Enzymes for industrial and household use (e.g., detergent enzymes) also must comply with product safety criteria, although the requirements are less stringent than in food, feed, veterinary, and medical applications.

## OTHER PRODUCTS AND APPLICATIONS

### Secondary Metabolites

Although solid-state surface culture technology was employed in the earliest attempts at large-scale production of penicillin, submerged culture is now the only method used in commercial production of antibiotics. Nevertheless, promising research on solid-state production methods continues, albeit at a slow pace. Solid-state production of oxytetracycline by *Streptomyces rimosus* grown on corncob has been described (53). Cephalosporins have been produced using *Cephalosporium acremonium*, *Streptomyces clavulgerus*, and *Aspergillus chrysogenum*. *Streptomyces cinnamomensis* has been used to produce monensin, an anticoccidial agent for poultry. Other potentially antibiotic-producing *Streptomyces* that have been cultured in solid state include *S. badius*, *S. aureofaciens*, and *S. flavovirens* (1). Studies of penicillin production by several strains of *Penicillium chrysogenum* have shown that the best producers in submerged culture are not necessarily the best in solid-state culture (54). Improving productivity demands selection of strains suited specifically to solid-state fermentation.

Solid substrate fermentation for producing gallic acid used in tanning, printing, and other applications has been described (13,14). Koji technology is employed in citric acid production in Japan (16). In producing citric acid by *Aspergillus niger*, Pallares et al. (45) noted that solid-state culture had doubled the productivity ( $\text{g L}^{-1} \text{ d}^{-1}$ ) of submerged culture, reducing the fermentation time from 14 to 6 days. Medium-impregnated polyurethane foam cubes were used as substrate (45). Productivity was sensitive to substrate particle size; 0.3-cm cubes gave the best results (45). Other products that have been produced by solid-state culture include several mycotoxins (14,46,55) and ergot alkaloids from various fungi, gibberellic acid from *Gibberella fujikuroi* (56,57), and kojic acid (14). In one case involving production of gibberellic acid and proteases, the yield of products was improved by fed-batch strategies (57), but nutrient feeding of solid-state batches other than at start-up remains a rare practice.

Ethanol has been commercially produced by aerobic–anaerobic solid-state fermentation of sweet sorghum grains in Taiwan (24). Production of  $\gamma$ -linolenic acid, a polyunsaturated fatty acid, by *Cunninghamella japonica* grown on rice and millet has been reported (58). The fungus *Monascus purpureus* or *Monascus anka* is used to com-

mercially produce a bright red polyketide pigment for food coloring and flavoring in Asia (41). An additional and developing application of solid-state fermentations is in producing fungal spores for use in a variety of biotransformations (1,14). Use of phytopathogenic and entomopathogenic fungi as herbicidal and insecticidal biocontrol agents promises further applications of solid-state fermentation technology for spore production (14,59–61).

### Environmental Remediation

Because of their ability to degrade otherwise persistent toxic organics, white rot fungi—for example, *Phanerochaete chrysosporium*, *Phanerochaete sordida*, and *Trametes versicolor*—are being tested for bioremediation of soils contaminated with polychlorinated biphenyls, pentachlorophenol, and other polycyclic aromatics (62). In the preferred mode of implementation, solid-state cultured fungal carriers such as corncobs, wood chips, and straw are mixed with the soil in situ. Commercial implementation requires, among other factors, the ability to economically and consistently produce large inocula. Solid substrates enriched with nutrients and concentrates of fungal spores, and formulated into pellets, are being developed to improve consistency of performance (62). Other pollution remediation applications of solid-state fermentations are composting (22,33,63), anaerobic digestion for producing methane in slurry fermentations and landfills (64–66), and treatment of gaseous pollutants and vapors in biofilters (65).

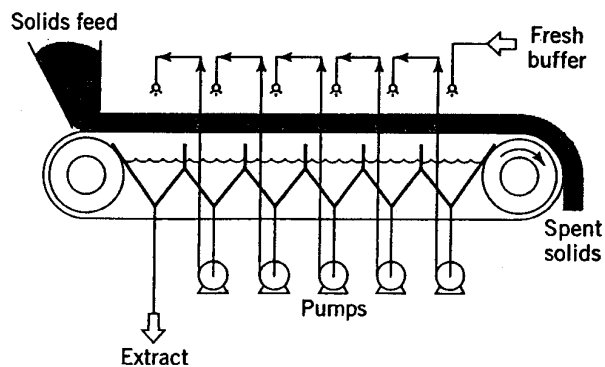
### Animal Feeds

Solid substrate fermentation methods are used to enrich many wastes and low-grade substrates for animal feed (1,11,12,22,25,46,65,67). One such widely used process is ensiling. Ensiling is used to stabilize and preserve forage crops; any nutrient enrichment is incidental. Typically, crops are ensiled immediately on harvest. An uncontrolled anaerobic primary fermentation by naturally occurring or inoculated (68,69) lactic acid bacteria takes place. Sugars present in the fodder are converted to lactic and acetic acids, causing a drop in pH. A low pH suppresses secondary fermentation by *Clostridia* that leads to butyric acid and spoilage. A good silage should generally have a pH of 4.0 to 4.2 and a butyric acid content of less than 5% of dry weight. A high dry matter content, for example, 20–30% w/w, of biomass at ensiling is preferred because secondary fermentation is difficult to suppress when dry solids are less than 15% w/w. Unlike *Clostridia*, the lactic acid bacteria remain active up to 70% w/w dry matter. Microbiology of silage has been detailed by Whittenbury (70) and Fenlon et al. (68). Silage inoculants have been described by Seale (69). Certain silages may contain animal and food processing waste (e.g., cattle and poultry manure, fish waste, or offal) and grain (e.g., corn), in addition to grass and other fodder crops. Fish silage has been described by Wignall and Tattersson (71); in-depth treatments of silage fermentations have been reported by McDonald (72) and Woolford (73).

### PRODUCT RECOVERY FROM FERMENTED SOLIDS

Unless the fermented solids are the desired product, the solids must be further processed to recover the enzyme or other metabolite. Volatile products such as ethanol may be recovered by direct distillation of the fermented mash (24). Most other products require extraction from fermented solids. The solids may be extracted moist or dry (44); moisture content can affect the extraction behavior. Because drying generally improves keeping quality, dried material from several batches can be accumulated for later extraction. The solid cake should be crumbled or otherwise size reduced for improved extraction. Either batch or continuous extraction may be employed. When feasible, continuous extraction with countercurrent flow of solids and solvent allows greater product recovery in a more concentrated solution. A suitable continuous extraction device is the Chisti contactor shown in Figure 8. Leaching machinery developed for the chemical industry can also be potentially adapted for use with fermented solids. A series of mixer-settlers has been used in small-scale extractions (74), but that arrangement is too cumbersome for commercial practice. Between two and five contacting stages are generally sufficient, but a larger number may be practicable with high-value products.

Cumulative contact periods typically do not exceed 50 min. Water, aqueous buffers, or dilute solutions of salts (e.g., 1% sodium chloride) or glycerol (e.g., 1% glycerol) are used to leach enzymes (74). Aqueous solutions of ethyl acetate, acetone, ethanol, or chloroform may be needed for nonprotein products such as most antibiotics and mycotoxins. In one case, aqueous ethanol (10%) was the best solvent for extracting gibberellic acid from fermented wheat bran (74). Pure methylene chloride has been used to extract mycotoxins (1). A suitable solvent must be selected empirically for each application. Factors that affect solvent choice are cost, toxicity, flammability, corrosion effects, disposal issues, solubility and stability of product, solubilities of other solutes, and compatibility with end use. The



**Figure 8.** Continuous countercurrent leaching of solids in an extractor. Solids are placed continuously on the stainless steel conveyor mesh (0.6 m wide, 6 m long) that moves at  $0.1\text{--}0.2\text{ m min}^{-1}$ . Fresh buffer is sprayed at the downstream end of the conveyor; the percolated extract is collected for extracting solids further upstream.

solvent-to-solid ratio generally ranges over 1 to 8; higher values increase the fraction of the product leached at the expense of producing a more dilute solution. Extraction temperature does not normally exceed 30 °C, but enzymes are commonly extracted at lower temperatures (e.g., 4–10 °C) to reduce denaturation, proteolysis, and microbial growth. Extraction performance and stability of the product may depend critically on pH. The pH of greatest stability may not be the same as that required for optimal leaching (75). Enzymes are generally extracted at pH values close to 7; however, to prevent precipitation, the extraction pH must not be the same as the isoelectric pH. Once the product has been extracted into solution, further purification employs the same well-known methods that are used in recovering extracellular products from submerged fermentations (27,42,43).

#### FERMENTATION-ENRICHED AND FERMENTATION-MODIFIED FOODS

Solid-state fermented foods occur worldwide. In some regions, such foods are the primary source of calories, proteins, and other nutrients for the consumers. Historically, fermented foods arose more by accident than by guided effort; nonetheless, recognition of the numerous advantages of the fermented products led to their established use. In comparison with the unfermented substrate, the fermented material may have one or more of the following characteristics: enhanced flavor, texture, and digestibility; reduced or eliminated undesirable compounds or flavors; protein enrichment or otherwise improved nutritional value; changed physical character; improved keeping quality; fast food quality of easier or quicker preparation; and other economic advantages such as reduced cooking time and hence lower energy demand. Table 4 notes some of the world's significant fermented products. Further details appear in the *Handbook of Indigenous Fermented Foods* (76), as well as other sources (6,8,40,41,77). The contribution of fermented products to the human food supply is likely to remain strong (78), and increasing cross-cultural spread is expected considering the popularity of tempe, tofu, and soy sauce in nonindigenous regions. The following sections discuss a small selection of the important foods as representative illustrations of the solid-state and solid substrate fermentation technologies.

#### Miso

Miso is a semisolid fermented soybean product that is widely consumed in Japan (18,40,41,79). Similar foods are produced in other parts of Asia (Table 4). In Japan miso is made into soup with added ingredients such as vegetables, meat, and seafood (18). Modern miso is generally produced from protein-rich yellow soybeans, but other varieties can be used. Cleaned soybeans are continuously washed, soaked overnight, drained, and cooked (Fig. 9). Cooking conditions vary. Large plants employ continuous pressure cookers with cooking done at 1.3–2.0 kg cm<sup>-2</sup> for 2–7 min (18). Cooked beans are ground using a meat grinder with 5-mm-diameter holes (18) before being mixed with separately produced koji for fermentation. Barley koji is used

in making "rural miso"; commercial processes employ rice koji (18). Miso koji is made from milled, cleaned, and usually cracked rice that is washed and soaked overnight in water at approximately 15 °C (18). Soaked rice is drained (1 h) and steamed. A horizontal belt steamer (Fig. 10) is commonly used. Steamed rice is unloaded onto a cooling conveyer, where a stream of air is blown through the rice layer to lower the temperature to 35 °C (18). Rice is then inoculated with the starter culture (called *tane-koji* in Japanese) of *Aspergillus oryzae*. Tane-koji is an olive-green, sporulated culture that has been grown on cooked rice mixed with a small amount of wood ash (18). Tane-koji is produced under aseptic and strictly controlled conditions. One kilogram of tane-koji is sufficient to inoculate 1,000 kg of rice (18). Many varieties of tane-koji are available with different capabilities for hydrolyzing proteins, carbohydrates, and lipids (18). An appropriate variety should be selected with regard to the amounts of proteins, oils, and carbohydrates in the mix of miso substrates (18).

Inoculated rice is transferred to a koji fermentor. Either a tunnel fermentor (Fig. 3) or, in larger plants, a rotary fermentor (Fig. 4) is used. The tunnel fermentor is mixed twice during the fermentation, but the rotary machine is mixed only once. Fermentation occurs at 30 °C for 40 h (18). Rice koji is then harvested and mixed with ground beans, salt, and a miso starter. The starter consists of halophilic yeasts, *Zygosaccharomyces rouxii* and *Candida versatilis*, and a halophilic lactic acid bacterium, *Pediococcus halophilus* (18). Fermentation is carried out in wooden vats, or tanks made of stainless steel or fiberglass-reinforced plastic. During fermentation miso is occasionally transferred from one vat to another to maintain homogeneity (18). The fermentation temperature is 25–30 °C (18). Ripened miso is taken out of vats, blended, pasteurized, and packaged. Process variations occur depending on the type of product desired (41).

The miso koji mold, *A. oryzae*, grows well around 30 °C (18), but optimal conditions for spore germination have been found to be 36 °C, ≥97% relative humidity, 0.1% carbon dioxide, and 20% oxygen (18). Optimal conditions for mycelial growth are 35–37 °C, ≥75% relative humidity, <1% carbon dioxide, and 20% oxygen (18). In practice, a lower than optimum temperature (28–30 °C) is used for germination, and 30–35 °C for cultivation in the koji chamber (18), apparently to avoid overheating. Desirable temperatures for production of enzymes are as follows: glucoamylase, 30 °C; α-amylase, 35 °C; protease, 25–30 °C; and carboxypeptidase, 35 °C (18). The optimal moisture level of cooked rice is 36–38%, and the optimal pH is 6.0–6.4 (18). Fermentation pH can be effectively maintained at 6–7 by supplementing the substrate with sodium glutamate or sodium succinate. Controlled pH favors higher-activity proteases (18). The microbiology and biochemistry of miso fermentation are further discussed by Abiose et al. (82).

#### Sake

Sake is a Japanese alcoholic beverage made from rice (10,40). Rice koji preparations for sake brewing are obtained by methods similar to those described for miso. The amount of tane-koji inoculum is 0.6–1 kg per kilogram of

Table 4. Solid Substrate Fermented Foods of the World

---

**Sufu.** A Chinese cheese made from soybean curd (*tofu*) by fermentation with molds such as *Actinomucor elegans* and *Mucor hiemalis* (41).

**Idli.** Small, white, steamed rice cake made by lactic acid fermentation of a thick batter of coarse-ground rice and fine-ground black gram in India. Idli cakes are moist, soft, and spongy, with a slightly sour flavor. Several varieties are produced. Other similar products of India are *dhokla* and *khaman*.

**Dosa.** Pancake made with thin pourable fermented batter of fine-ground rice and gram. Important part of diet in South India and Sri Lanka.

**Ambali.** A cooked product of fermented millet flour batter and rice. India.

**Puto.** Steamed cake of milled, acid-fermented rice. Similar to idli, but without legume. Important food in Philippines. Several varieties.

**Enjera.** Very flexible, soft, sour leavened pancake containing many small uniformly distributed gas bubbles. Made from thin, pourable fermented batter of *tef* (*Eragrostis abyssinica*) flour, or other ground grain (corn, sorghum, millet, barley, wheat). Consumed as staple in Ethiopia.

**Hopper or appa.** Steam-baked product made from acid-fermented dough (rice or wheat flour) formulated into liquidy batter with coconut water. Sri Lanka.

**Kisra.** Flat bread made from fermented sorghum flour batter in Sudan.

**Ogi** (also *furah* and other names). Fermented cereal flour (corn, sorghum, or millet) made into a slightly sour gruel. Important source of calories in Nigeria.

**Uji** (and other names). Suspension of cereal (corn, millet, sorghum) or cassava flour (relatively large particles) in water. Sour-acid fermented either before or after cooking. East Africa.

**Mahewu** (also *magou* or *mageu*). Suspension of acid-fermented corn flour used as high-calorie beverage/food. More dilute than uji or ogi. Cooked cornmeal in water is fermented after adding a small amount of wheat flour. Product is very slightly alcoholic. Produced in large commercial operations in South Africa. Similar cereal-derived alcoholic beverages/foods occur throughout sub-Saharan Africa.

**Sake.** Rice wine of Japan, and other similar products: *brem bali* (Indonesia); *mie-chiu* (China); *tapoi* (Philippines); *sato* (Thailand); *yakju* (Korea); and *sonti* (India). See text.

**Gari.** A dry granular starchy product of West Africa (80,81). Made into staple food by adding hot or cold water. Made from peeled, grated cassava by fermenting in cloth bags while squeezing out the water over 12–96 h. The anaerobic acid fermentation produces a sour taste. Fermented solids are sun dried and lightly toasted in iron pans. A small amount of palm oil may be added during toasting to impart a pale-yellow color. Solids are sieved and sold in markets. Very poisonous varieties of cassava may be used in making gari. Crushing of cassava releases naturally present linamarase, which breaks down the cyanogenic linamarin to reduce the cyanide content. Cyanide produced boils off as hydrogen cyanide gas, indicating the need for good ventilation during processing. Acid pH produced during fermentation enhances the action of linamarase (pH optimum of 5.5). In addition, microbial action may produce enzymes that enhance the breakdown of cyanoglycosides.

**Miso.** Fermented soybean paste of Japan. Other similar products: *jang* (China); *doenjang* and *kochujang* (Korea); *taucho* or *tauco* (Indonesia); *tao-tsi* (Philippines) (18,40,41,79,82). See text.

**Soy sauce.** Japanese *shoyu*, and similar products throughout Asia: *chiang-yu* (China); *han jang* (Korea); *kecap* (Indonesia) (9,40,41,76,79). See text.

**Natto.** Soaked and cooked soybeans fermented by bacteria, principally *Bacillus natto*, consumed in Japan. Other similar products: *thua-nao* of Thailand; *kinema* of Nepal, Bhutan, Sikkim, and parts of northeastern hills of India; *dagé*, an Indonesian produce made of bacterial fermented peanuts and other oilseeds.

**Tempe** (or *tempeh*) and similar products: *tempe kedele*, *tempe gembus*, *tempe benguk*, *tempe bonghrek*, *oncom*, or *ontjom*. See text. A Japanese solid food, *hamanatto*, is also made from soy beans fermented by fungi (41).

**Dawadawa.** Fermented African locust bean (*Parkia filicoidea*) product used as flavoring agent in West Africa (14).

**Kenkey** (and other similar products). Fermented corn dough used as staple in Ghana (8).

**Mawé.** Acid-fermented corn dough used in Benin and Togo (8).

**Pozol.** Fermented corn dough used as staple in parts of Mexico.

**Kocho.** Unleavened flat bread made from fermented pulp of ensete (*Ensete ventricosum*) or 'false banana' plant (a banana-like plant that does not bear bananas). Used as staple in certain regions of Ethiopia.

**Tapai.** Malaysian fermented glutinous rice or cassava gratings. Sweet and mildly alcoholic. Used as dessert. Similar products occur elsewhere: *lao chao* in China (41); *tapé ketan* and *tapé ketella* of Indonesia (40); and *peujeum* in Sudan.

**Nham.** Fermented minced raw pork similar to sausages. Thailand.

**Fermented seafood.** Usually fish or shrimp pastes, or sauces: *bagoong*, *patis* (Philippines); *ngapi* (Burma); *trassi*, *kccap ikan* (Indonesia); *belachan*, *budu* (Malaysia); *mam*, *nuoc-mam* (Vietnam); *jeotkal* (Korea); *prahoc* (Cambodia); *kapi* (Thailand); *padec* (Laos); *shottsuru*, *shiohara*, *katsuobushi* (Japan); *faseich* (Sudan) (14); *kobi* (Ghana) (14); and fermented fish of Northern Europe.

**Balao balao.** Fermented rice–shrimp mixture eaten in Philippines. Cooked, somewhat pasty rice is blended with fresh, unshelled, salted shrimps, and fermented for 7–10 days. Shrimp shell becomes quite soft. Product cooked before consumption.

**Burong dalag.** A fermented fish and rice product of the Philippines.

**Fermented milk products.** Yogurt; sour cream; cheeses; *ergo* and others (Ethiopia); *kefir*, *koumiss* (Russia); *dahi* (India); *tairu* (Malaysia); *laban rayeb*, *laban zeer*, *laban zabadi* (Egypt); *liban argeel*, *liban khather*, *mast*, *mass taw*, *shenina*, *dabbo* (Iraq).

**Kishk.** A fermented milk–wheat mixture of Egypt. Stored as dry balls.

**Trahanas** (and other names). Made from crushed wheat and fermented sheep milk in Greece, Turkey, and Cyprus. Stored as dry biscuits or dry crumbled solids.

**Fermented vegetables** (8). Sauerkraut (Europe, North America); Chinese sauerkraut *hum choy*; acid-pickled olives and cucumbers; various Indian and Malaysian pickles; various varieties of *kimchi* (Korea); kimchilike products of Thailand and elsewhere.

---

Source: Refs. 6, 76, and 77.

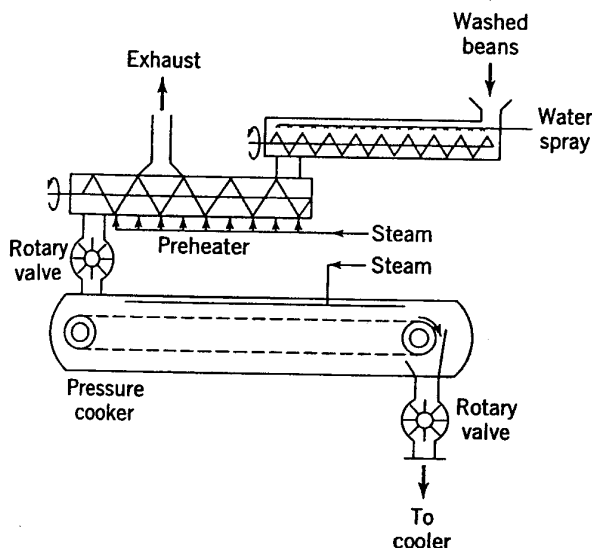


Figure 9. Continuous washing, soaking, preheating, and pressure cooking of soybeans.

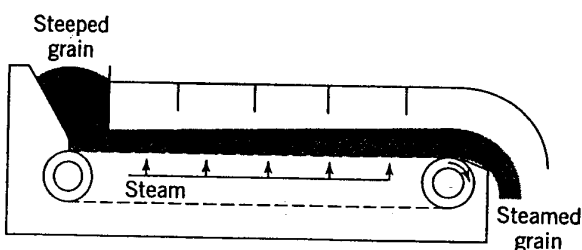


Figure 10. Continuous grain steamer.

the original rice, corresponding to about 2,000 fungal spores for each rice grain (10). The koji used is white because mold growth is arrested before sporulation (10). The unsporulated koji is relatively dry; the rice kernels are relatively hard and unclumped (10). The koji is then used in preparing the sake yeast starter mash, or *moto*. The traditional method of *moto* making required about a month-long fermentation of koji (300 kg) and water (1 m<sup>3</sup>) (10). A succession of microbial populations developed, including lactic acid bacteria that reduced the pH and created an environment in which only the sake yeast, *Saccharomyces cerevisiae*, thrived (10). In modern *moto* production, fermentation time is reduced by adding lactic acid to water (pH 3.5–3.8) before mixing with koji. A large inoculum of sake yeast and steamed rice is also added. In a few days the starch is hydrolyzed, and fermentation begins. The next step is a *moromi* fermentation; it involves further addition of steamed rice, koji, and water to *moto*. Three additions are made at 1-day intervals (10). Temperature is lowered at each addition to control contamination because the added materials dilute the acids and alcohol. The temperature of *moromi* is controlled carefully, and after the final addition the temperature is 7–8 °C (10).

Saccharification of starch and fermentation occur simultaneously in *moromi* (unlike in *moto*) until the alcohol content reaches nearly 20% v/v (10). Some steamed rice may be added during the final stages of fermentation to produce a sweeter product (10). Once the fermentation has almost ceased, alcohol is added to the mash to bring the concentration to 20–22% by volume (10). Solids are filtered out; the liquid product is further clarified, pasteurized, aged, blended, diluted to 15.0–16.5% v/v alcohol, and packaged (10). Unlike Western beer fermentations, sake mash is very high in solids that help to keep a large amount of yeast in suspension (10).

Beverages similar to sake are also found in China and many other Asian countries (Table 4). Other quite different indigenous alcoholic beverages, some made through solid substrate fermentation, are found in Africa and elsewhere (76). Some of these products are now produced in large industrial operations (77).

### Soy Sauce

Soy sauce originated in China, but it is widely produced and consumed throughout Southeast Asia, Japan, and, increasingly, Europe and North America. The history, variety, and production technology of soy sauce have been detailed by Fukushima (9) and others (40,41,76,79). Here, a short description of only the latest, large-scale Japanese production methods is given.

Manufacture of soy sauce (*shoyu* in Japanese) involves a solid substrate koji fermentation, a brine or *moromi* fermentation, and refining steps (40,41,79). In koji making, soybeans or, more usually, defatted soybean flakes or grits are moistened and cooked in continuous pressure cookers (Fig. 9). The latest cooking methods employ a cooking time of 0.25 min or less, at approximately 7 kg cm<sup>-2</sup> gauge pressure, equivalent to 170 °C (9). Rapid high-temperature cooking reduces loss of proteins and improves the yield of hydrolyzed amino acids in the final product. Cooked beans are mixed with roasted wheat that has been cracked into four or five pieces (9). Wheat roasters operate in a continuous mode at 170–180 °C (9). The ratio of wheat to beans is commonly 1:1, but it varies with the variety of *shoyu* (9). The mixed substrate is inoculated with a pure culture of *Aspergillus oryzae* (or *A. sajae*). The fungal spore density at inoculation is about  $2.5 \times 10^8$ /kg of wet solids (1). After a 3-day fermentation the substrate mass becomes green-yellow because of sporulation (9). Too long an incubation causes excessive, unwanted sporulation, production of ammonia from deamination of amino acids, and off-flavors in the final product (41). Koji is now harvested for use in the *moromi* step. Koji production is highly automated and continuous (9,18). Among the largest of koji-making processes is that of Kikkoman Corporation; it produces up to 4,150 kg h<sup>-1</sup> koji, which is equivalent to processing 1,650 kg h<sup>-1</sup> raw soybeans and 1,775 kg h<sup>-1</sup> wheat (9).

Harvested koji is mixed with brine containing at least 20% salt for the *moromi* fermentation step (9,41). Large outdoor tanks are used for *moromi* fermentation. A specially selected culture of lactic acid bacteria is added to *moromi* mash, and the temperature is kept relatively low (15–20 °C) for the first month or so (9). Low temperature

prevents a too rapid growth and attendant rapid decline in pH. A pH drop that is too rapid causes undesirable early arrest of the proteolytic activity of koji enzymes; action of koji glutaminase is also obstructed (9). When the pH of moromi has declined to pH 5, a separately grown culture of the yeast *Zygosaccharomyces rouxii* is added (9). The temperature is then gradually raised to nearly 30 °C until a vigorous alcoholic fermentation occurs (9). On cessation of the alcoholic fermentation, the temperature is again reduced to about 25 °C and maintained there for the last 2 months (9). Aged moromi is filtered using automatic filter presses; the liquor is pasteurized in continuous flow plate heat exchangers and packaged in plastic bottles (9). The production schemes vary a great deal within Japan, and many quite primitive methods are encountered elsewhere in Asia. Chinese practices have been outlined by Wood and Min (41).

### Tempe

Tempe (or tempeh) kedele is a fermented soybean product consumed in Indonesia, Malaysia, Singapore, the Netherlands, the West Indies, and, increasingly, the United States and Canada (8,40,41,76). Tempe kedele is made from partly cooked soybeans that are fermented with the mold *Rhizopus oligosporus*. The mold mycelium completely covers, penetrates, and binds the beans into a cake. In addition to the beans, lower-quality tempe varieties may contain solids such as cassava grits, soybean coats, or tofu that are added before fermentation. Freshly fermented tempe is creamy white; it has a mushroomlike flavor that becomes nutty and peppery on deep frying (8,76). Raw tempe does not keep well. Unless consumed within 24–48 h, it should be deep fried, dried, or blanched and refrigerated (76).

Production processes vary a great deal. In typical cottage-level processing in Indonesia, cleaned dry soybeans are washed and partially cooked by boiling for 2.5–3 h. An overnight soaking in clean water follows (8). A bacterial acid fermentation occurs and the pH drops to 4.5–5.3. The beans are now dehulled by rubbing with hands or feet. Hulls float on top and are washed away. Well-drained beans are inoculated using a previous batch of sporulated tempe that may have been sun dried. Alternatively, mold grown and dried on large leaves of *Hibiscus tiliaceus* may be used (8,76). Inoculum densities of 10<sup>7</sup> spores (or 10<sup>7</sup> cfu) per kilogram of wet solids have been reported (1,8). Overinoculation may produce excessive heat that inactivates fungal mycelium; *Bacillus* species proliferate, causing spoilage (8). Similarly, suboptimal inoculum density may cause outgrowth of yeasts and bacteria and again spoilage (8). Inoculated beans are lightly packed in relatively flat packets of *Hibiscus*, banana, or other broadleaves for 30–40 h of fermentation at ambient conditions (76). Similar general schemes are followed in large-scale production processes operated in the United States, Asia, and other countries; exceptions are mechanized processing, better-defined inoculum, and fermentation in perforated stainless steel trays (2- to 3-cm-deep bean layer) or perforated plastic bags (76). Fermentation is nonsterile; the bacterium *Klebsiella pneumoniae* that is invariably present in com-

mercial preparations leads to vitamin B<sub>12</sub> enrichment of the product (76).

Studies on production of *R. oligosporus* spores (pure culture) for commercial tempe inoculum indicate that viable spore counts of 10<sup>9</sup>–10<sup>10</sup>/kg dry substrate can be obtained on a variety of grains (1). Spores may be freeze-dried, but typically about one-log reduction in viability accompanies drying (1). Freeze-dried spores generally keep for 6 months (1).

Other tempelike products are consumed in Indonesia, including *tempe gembus*, made from waste produced by soybean curd factories; *tempe benguk*, produced using the legume *Mucuna pruriens* instead of soybeans; *tempe mata kedele*, made from soybean hulls; and *tempe bongkrek*, made from coconut press cake. The latter type can be potentially contaminated with the bacterium *Pseudomonas cocovenenans*, which produces lethal toxins (76). *P. cocovenenans* suppresses the growth of the tempe mold; hence, tempe bongkrek must never be consumed if a luxuriant growth of the mold has not developed (76). Formation of bacterial toxins is suppressed below pH 6, and an incubation temperature of 37 °C or higher prevents development of *P. cocovenenans* (76). The mold that is primarily responsible for most tempe fermentations is *Rhizopus oligosporus*, but other *Rhizopus* species, such as *R. oryzae*, *R. achlamydosporus*, and *R. arrhizus*, as well as non-*Rhizopus* fungi, may be involved (8).

### Oncom

Oncom (or ontjom) is another tempelike product made from peanut press cake in Indonesia (41,76). *Rhizopus* species are used to produce the black oncom (*oncom hitam*), whereas the preferred red oncom (*oncom merah*) is produced using *Neurospora* species (76). *Neurospora sitophila*, *N. crassa*, and *N. intermedia* are typical oncom molds (76). In quite different processes, *N. sitophila* has been investigated also for protein enrichment of low-grade substrates such as sugar cane bagasse for animal feed (11).

### Sausages

Fermented meat products such as bologna, summer sausage, thuringer, teewurst, dry sausage, pepperoni, and salami are produced by solid-state anaerobic fermentations utilizing acid-forming bacteria, particularly *Lactobacillus*, *Pediococcus*, and *Micrococcus* species. Typically, ground meat is mixed with curing agents and a starter culture at low temperature (–1 °C or less), and placed in cloth or other casings. Fermentation occurs at 20–40 °C. In dry sausage production in the United States, for example, a fast acid fermentation is employed to prevent growth of spoilage microorganisms. The curing mixture is predominantly salt and nitrite. Nitrite reacts with myoglobin in meat to produce the characteristic dark-red color of the product. Either *Pediococcus acidilactici* or *P. pentosaceus* is used for starter, depending on the selected fermentation temperature. Slower fermentation at 24 °C is commonly used in Europe. The curing mixture consists of salt, nitrate, and other spices. Naturally present micrococci and staphylococci convert the nitrate to nitrite for the desired coloring effect. After the lactic acid fermentation, a further incu-

bation period of up to 7 days at low temperature is practiced, with the meat held in 0.2-m-deep layers in metal pans.

### Coffee

Coffee, after oil, is the second largest traded commodity, and more than 1.2 billion cups are consumed daily (83). Coffee processing employs a solid substrate fermentation for removing adhering mucilage (mesocarp) from ripe, mechanically pulped coffee cherry. This method is used predominantly only for *Coffea arabica* (84), which supplies the high-quality end of the market (83). Apparently, fermentation is not essential to flavor development, serving merely to remove the mesocarp from the parchment that encloses the bean. Nevertheless, the fermentation needs to be carefully conducted, or off-flavors develop that affect the final cup quality (84). In the preferred mode of fermentation, pulped cherry is placed in concrete or wooden tanks arranged for continuous drainage of the liquors produced (84). An alternative fermentation scheme utilizes under water fermentation with or without continuous water flow (84). The fermentation lasts 20–100 h, depending on factors such as pH, the coffee variety and stage of ripeness, aeration, and concentration of ions (for example,  $\text{Ca}^{2+}$  enhances the activity of certain enzymes) (84). Once the mucilage has degraded, the solids are washed and graded by water in concrete channels, and dried to produce parchment coffee (84). Milling (dehulling) removes the parchment to yield green beans used in commerce. The well-known coffee grounds are obtained by crushing blended and roasted green beans. Further processing is required to produce the instant varieties of coffee (83).

### Cocoa

Cocoa beverages and chocolate are made from fermented cocoa (*Theobroma cacao*) seed or beans (85). Mature cocoa fruits or pods are harvested and split open to release the pulp-enveloped seeds. The pulp ferments as the seeds are piled in 2- to 4-ft-diameter heaps placed on perforated banana leaves that allow drainage of the liquids released (14,85). Alternative fermentation schemes employ perforated wooden boxes and other methods (85,86). The fermentation usually lasts 2–8 days, depending on the cocoa variety. The cocoa mass is turned several times during fermentation to ensure aeration and homogeneity and to avoid clumping (85,86). Turning also discourages mold growth on the better-aerated, exposed parts (86). A succession of microorganisms develop, including yeasts that are followed in turn by lactic acid producers, acetic acid bacteria, and, finally, aerobic spore-forming bacteria (86). The heat developed during the two-stage anaerobic-aerobic fermentation kills the cocoa embryo. Fermentation degrades the pulp, making removal easier, and products of fermentation penetrate the beans, stimulating complex biochemical reactions that are essential to flavor development (85,86). Fermented beans are dried, cleaned, and sold to chocolate processors or marketing organizations.

### Mushrooms

The variety of edible mushrooms is tremendous, but only a few types are commercially cultivated. Production of

mushrooms, mushroom compost and other substrates, and mushroom spawn utilize solid substrate fermentation methods that are comprehensively described elsewhere (2–4).

### SAFETY CONSIDERATIONS

Microorganisms used in solid substrate fermentations have the potential to produce harmful effects in humans and animals. The history of occupational disease in solid-state cultivation is reflected in names such as cheese-maker's lung and mushroom worker's lung (87). Allergic reactions and immunological hypersensitivity have been associated with exposure to fungal spores, including those of *Aspergillus niger*, *Aspergillus oryzae*, *Aspergillus awamori*, and *Aspergillus batatae* in industrial settings (88,89). Conditions such as dermatitis, asthma, vasomotor rhinitis, and itchy skin have been observed among exposed workers (87,88). Exposure to *Bacillus thuringiensis* has produced similar effects in humans (88). *Aspergillus* and *Pseudomonas* species have caused fatal infection in immunocompromised individuals and serious lung infection in cystic fibrosis patients (89). Fatal infections with *Aspergillus fumigatus* in previously healthy adults have been observed (89).

Inhalation of spores of edible mushrooms has long been associated with disease among mushroom growers (87). Allergic reactions to spores of *Pleurotus* species, *Lentinus edodes* (3), and others have been documented. *Pleurotus* spores survive for several days after inhalation in the human respiratory tract, suggesting the potential for germination in the body (3). Allergic reactions encountered in cultivation of *Agricus* species are linked to other molds that inhabit the substrate (3). In best-practice cultivation, attention to handling and processing of mushroom substrate and product should accompany ventilation of work areas and the use of personnel protective clothing.

In addition to microbes, fermentation products such as antibiotics and enzymes have been implicated in occupational asthma (87,89). Severe allergic reactions to *Bacillus subtilis* proteases are well known (89). Under certain environmental conditions, organisms such as *Aspergillus flavus* and *Aspergillus oryzae* are known to produce lethal toxins (89). Specific strains of the blue-veined cheese mold, *Penicillium roqueforti*, also produce mycotoxins under narrowly defined environmental conditions (7). Species of the genus *Claviceps* and some members of other genera produce toxic ergot alkaloids. Some other toxin-producing fungi are noted in Table 5. Potentially, most physiologically active fermentation products can be disruptive to health, and certain products are highly toxic. Aflatoxins are potent carcinogens. For a given fungus, the product spectrum often depends on the fermentation conditions (temperature; pH; moisture content; type of substrate; agitation; metabolic energy source; amounts of oxygen and carbon dioxide; and nature and concentration of micronutrients, metal ions, and other chemicals); hence, evaluations under relevant conditions are essential. In particular, information from submerged culture may be an unsatisfactory indicator of behavior in solid-state fermentation.

**Table 5. Toxin-Producing Fungi**


---

<i>Aspergillus alliaceus</i>
<i>Aspergillus clavatus</i>
<i>Aspergillus melleus</i>
<i>Aspergillus niger</i>
<i>Aspergillus ochraceus</i>
<i>Aspergillus parasiticus</i>
<i>Aspergillus sclerotiorum</i>
<i>Aspergillus sulphureus</i>
<i>Chaetomium cellulolyticum</i>
<i>Fusarium moniliforme</i>
<i>Fusarium graminearum</i>
<i>Penicillium islandicum</i>
<i>Penicillium roqueforti</i>
<i>Penicillium viridicatum</i>

---

Handling of the substrate used in solid substrate fermentation requires care. A seemingly innocuous substrate may be contaminated with microbial spores or may generate allergenic dust. Raw material for composting may be heavily contaminated with human and animal pathogens, including eggs of parasites. Hence, certain high temperatures and holding time combinations are essential in composting to ensure destruction of pathogens and parasites. Depending on the composting process and the end use of the product, a uniform maximum temperature of 55 °C maintained for 3–15 days may be considered acceptable (33). Special care in process design and operation may be necessary to prevent recontamination of the final product. Cases of such contamination do occur perennially (e.g., infection from vegetables fertilized with manure; *Listeria* contamination in cheese). Sometimes inadequacies in processing may lead to a dangerous product: cases of cyanide poisoning from consumption of insufficiently processed *gari* occur from time to time in West Africa. Normally, fermentation and other process steps in *gari* making should substantially eliminate the cyanogenic glycosides (mostly linamarin and lotaustralin) that occur naturally in cassava tubers. Cases of fatal poisonings from consumption of poorly prepared *tempe bongkerk* have occurred in Indonesia.

Poor operational practice and failings in process and plant design can exacerbate the hazard. Experience suggests the following guidelines for minimizing process risks: (1) the number of operators should be kept to a minimum through mechanization and automation; (2) personnel-product contact should be minimal to reduce contamination of the product and ensure safety of operators; (3) operations should be enclosed and should be designed to minimize generation of aerosols and particulates; (4) the applicable GMP standards for food and pharmaceutical products should be followed; (5) the environment in and around the process plant must meet acceptable workplace air quality standards, especially for particulates, microorganisms, spores, humidity, and temperature; (6) the process equipment and the facility (including the air conditioning system) must be frequently sanitized; (7) facility design and operation must eliminate the risk of recontaminating the processed product; (8) there should be checks on the quality of the in-process material and the final prod-

uct; and (9) satisfactory disposal of solid, liquid, and gaseous wastes must be ensured.

Operators should be fully informed of risks, and they should be given the training necessary for safe processing. Where required, personnel protective equipment should be provided. There should be adequate supervision, including routine health surveillance. Personnel who are ill, and those with open sores and cuts, should be sent home or moved to other, noncritical work. Attention to protection of peripheral support staff, for example, maintenance and cleaning personnel, is especially important because they may not have the knowledge or training for the potential risks. Thus, for example, trained, regular process operators should clean and sterilize a bioreactor before maintenance work.

The solid substrate fermentation facility should be designed and operated to prevent cross-contamination between dirty and clean areas. This goal requires attention to plant layout; movement of personnel, in-process material, process machinery; and flow of air in the facility. A minimum 12-Pa (0.05-in water gauge) pressure differential is recommended between adjacent clean and dirty areas. Negative pressure with respect to surroundings should be maintained in most solid-state fermentation chambers. Alternatively, the fermentation chamber may be under positive pressure with respect to the adjacent area, but that area should be under negative pressure with respect to other surrounding areas. Most work areas should comply with a relative humidity standard of 40–50%. Relative humidities lower than 40% cause problems with static electricity, whereas values greater than 50% promote corrosion. Between 10 and 15 air changes per hour are recommended for most plant areas. Workspaces for substrate preparation, downstream processing, and fermentation should attain 15–20 air changes per hour. Generally, conditioned air should be supplied at the ceiling level and return at lower wall locations. Preferably, particle counts should not exceed  $3.5 \times 10^6 \text{ m}^{-3}$  ( $10^5$  per cubic foot) for particles 0.5  $\mu\text{m}$  or larger, and  $2.5 \times 10^4 \text{ m}^{-3}$  (700 per cubic foot) for particles 5.0  $\mu\text{m}$  or larger. There should be no more than 25 cfu/10 ft<sup>3</sup> of air. Air entering and exiting the fermentation chamber should be filter sterilized with 0.45- $\mu\text{m}$  (or better) absolute filters that are also capable of in situ steam sterilization. A filter maintenance and replacement program should be in place. The practice of sterilizing solid substrate aeration gas by bubbling it through caustic or acid baths is misguided. Admittedly, the standards recommended here are not intended for processes such as composting and mushroom cultivation, but for plants producing foods, food additives, bulk pharmaceuticals, and secondary metabolites. The recommended practices are based on the author's experience. Note that the standards for packaging, sterile processing, and final dosage pharmaceuticals processing areas are significantly higher than the ones given here (90,91).

Much of the indigenous fermented food production in Asia, Africa, and South America is carried out at the cottage- or village-scale level with little consideration for hygienic processing. With few exceptions, even the larger plants do not come close to meeting the minimum standards expected in the developed world. The practices rec-

ommended in this review are for best-case production processes in the developed world where attention to hygiene and safety have transformed the traditional processing methods. Finally, as examples of toxic gari and tempe bongkerk prove, a thorough understanding of the fermentation microbiology and biochemistry is essential if disasters are to be prevented (78). Such knowledge is indispensable to developing large-scale production processes and in gaining increased acceptance of products beyond the regions of origin.

#### BIBLIOGRAPHY

- R.E. Mudgett, in A.L. Demain and N.A. Solomon eds., *Manual of Industrial Microbiology and Biotechnology*, American Soc. for Microbiology, Washington, D.C., 1986, pp. 66–83.
- T.E. Tautorius, *Adv. Biotechnol. Processes* 5, 227–273 (1985).
- F. Zadrazil, D. Ostermann, and G.D. Compare, in H.W. Doelle, D.A. Mitchell, and C.E. Rolz eds., *Solid Substrate Cultivation*, Elsevier, London, 1992, pp. 283–319.
- W.A. Hayes and N.G. Nair, in J.E. Smith and D.R. Berry eds., *The Filamentous Fungi*, vol. 1, E. Arnold, London, 1975, pp. 212–248.
- W. Röcken and P.A. Voysey, *J. Appl. Bacteriol. Symp. Suppl.* 79, 38S–48S (1995).
- A.H. Rose, ed., *Fermented Foods*, Academic Press, London, 1982.
- B.A. Law, in A.H. Rose ed., *Fermented Foods*, Academic Press, London, 1982, pp. 147–198.
- F.M. Rombouts and M.J.R. Nout, *J. Appl. Bacteriol. Symp. Suppl.* 79, 108S–117S (1995).
- D. Fukushima, in K.H. Steinkraus ed., *Industrialization of Indigenous Fermented Foods*, Dekker, New York, 1989, pp. 1–88.
- K. Yoshizawa and T. Ishikawa, in K.H. Steinkraus ed., *Industrialization of Indigenous Fermented Foods*, Dekker, New York, 1989, pp. 127–168.
- M. Moo-Young, Y. Chisti, and D. Vlach, *Biotechnol. Adv.* 11, 469–479 (1993).
- U.C. Banerjee, Y. Chisti, and M. Moo-Young, *Resour. Conserv. Recycl.* 13, 139–146 (1995).
- L.M. Miall, in J.E. Smith and D.R. Berry eds., *The Filamentous Fungi*, vol. 1, E. Arnold, London, 1975, pp. 104–121.
- K.E. Aidoo, R. Hendry, and B.J.B. Wood, *Adv. Appl. Microbiol.* 28, 201–237 (1982).
- K. Bosecker and M. Kürsten, *Process Biochem.* 13, 2–4 (1978).
- E. Cannel and M. Moo-Young, *Process Biochem.* 15, 2–7 (1980).
- L.A. Underkofler, *Chem. Eng. Prog. Symp. Ser. No. 69* 62, 11–20 (1966).
- H. Ebine, in K.H. Steinkraus ed., *Industrialization of Indigenous Fermented Foods*, Dekker, New York, 1989, pp. 89–126.
- B.K. Lonsane, N.P. Ghildyal, S. Budiartman, and S.V. Ramakrishna, *Enzyme Microb. Technol.* 7, 258–265 (1985).
- P. Weiland, in F. Zadrazil and P. Reiniger eds., *Treatment of Lignocellulosics with White Rot Fungi*, Elsevier, London, 1988, pp. 64–76.
- E. Cannel and M. Moo-Young, *Process Biochem.* 15, 24–28 (1980).
- M. Moo-Young, A.R. Moreira, and R.P. Tengerdly, in J.E. Smith, D.R. Berry, and B. Kristiansen eds., *The Filamentous Fungi*, vol. 4, E. Arnold, London, 1983, pp. 117–144.
- C.W. Hesseltine, *Process Biochem.* 12, 29–32 (1977).
- R.P. Tengerdly, *Trends Biotechnol.* 3, 96–99 (1985).
- A. Durand and D. Chereau, *Biotechnol. Bioeng.* 31, 476–486 (1988).
- Y. Chisti, *Airlift Bioreactors*, Elsevier, London, 1989.
- Y. Chisti and M. Moo-Young, in V. Moses and R.E. Cape eds., *Biotechnology: The Science and the Business*, Harwood, New York, 1991, pp. 167–209.
- Y. Chisti and M. Moo-Young, *Trans. I. Chem. E.* 74A, 575–583 (1996).
- Y. Chisti, *Chem. Eng. Prog.* 88, 55–58 (1992).
- Y. Chisti and M. Moo-Young, *J. Ind. Microbiol.* 13, 201–207 (1994).
- B.K. Lonsane, G. Saucedo-Castaneda, M. Raimbault, S. Roussos, G. Viniegra-Gonzalez, N.P. Ghildyal, M. Ramakrishna, and M.M. Krishnaiah, *Process Biochem.* 27, 259–273 (1992).
- B.A. Prior, J.C. Du Preez, and P.W. Rein, in H.W. Doelle, D.A. Mitchell, and C.E. Rolz eds., *Solid Substrate Cultivation*, Elsevier, London, 1992, pp. 65–85.
- E.I. Stentiford and C.M. Dodds, in H.W. Doelle, D.A. Mitchell, and C.E. Rolz eds., *Solid Substrate Cultivation*, Elsevier, London, 1992, pp. 211–246.
- M. Gutiérrez-Rojas, S.A.H. Hosn, R. Auria, S. Revah, and E. Favela-Torres, *Process Biochem.* 31, 363–369 (1996).
- B.L. Rathbun and M.L. Shuler, *Biotechnol. Bioeng.* 25, 929–938 (1983).
- S. Solis-Pereyra, E. Favela-Torres, M. Gutiérrez-Rojas, S. Roussos, G. Saucedo-Castañeda, P. Gunasekaran, and G. Viniegra-González, *World J. Microbiol. Biotechnol.* 12, 257–260 (1996).
- Y. Chisti, *Biotechnol. Adv.* 11, 385 (1993).
- A. Pandey, *Process Biochem.* 27, 109–117 (1992).
- P.W. Lambert, in J.E. Smith, D.R. Berry, and B. Kristiansen eds., *The Filamentous Fungi*, vol. 4, E. Arnold, London, 1983, pp. 210–237.
- K.H. Steinkraus, in J.E. Smith, D.R. Berry, and B. Kristiansen eds., *The Filamentous Fungi*, vol. 4, E. Arnold, London, 1983, pp. 171–189.
- B.J.B. Wood and Y.F. Min, in J.E. Smith, and D.R. Berry eds., *The Filamentous Fungi*, vol. 1, E. Arnold, London, 1975, pp. 265–280.
- Y. Chisti and M. Moo-Young, *I. Chem. E. Symp. Ser.* 137, 135–146 (1994).
- K. Aunstrup, in L.B. Wingard, Jr., E. Katchalski-Katzir, and L. Goldstein eds., *Applied Biochemistry and Bioengineering*, vol. 2, Academic Press, New York, 1979, pp. 27–69.
- B.K. Lonsane and N.P. Ghildyal, in H.W. Doelle, D.A. Mitchell, and C.E. Rolz eds., *Solid Substrate Cultivation*, Elsevier, London, 1992, pp. 191–209.
- J. Pallares, S. Rodríguez, and A. Sanromán, *Bioprocess Eng.* 15, 31–33 (1996).
- C.W. Hesseltine, *Biotechnol. Bioeng.* 14, 517–532 (1972).
- A. Singh and P. Mishra, *Microbial Pentose Utilization: Current Applications in Biotechnology*, Elsevier, Amsterdam, 1995, pp. 71–98.
- M.A. Millett, A.J. Baker, and L.D. Satter, *Biotechnol. Bioeng. Symp.* 5, 193–219 (1975).
- M. Becerra and M.I. González Siso, *Enzyme Microb. Technol.* 19, 39–44 (1996).
- V.K. Garg, M.A.C. Costello, and B.A. Czuba, in S. Seetharam and S.K. Sharma eds., *Purification and Analysis of Recombinant Proteins*, Dekker, New York, 1991, pp. 29–54.