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Fungi and Biotechnology

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THE INDUSTRIAL SIGNIFICANCE OF FUNGI

The term **fungal biotechnology** is often reserved for modern industrial processes involving genetically modified organisms, but a broader reading includes the baking and brewing practices that originated in the ancient world. Humans have employed fungi for thousands of years. For most of our history this was unconscious: people followed preparative methods that produced the desired result and knew nothing about yeasts that made dough rise and fermented cereals. Mushroom cultivation on logs and horse dung is another example of early biotechnology that has flavoured the omnivorous diet of our species for generations. Methods for controlling fungal fermentations have developed in parallel with advances in the field of microbiology since the nineteenth century. The use of fungi to produce antibiotics and other pharmaceutical products is a more recent part of this endeavour and the introduction of molecular genetic manipulation of fungal strains has revolutionised the business of biotechnology. It is important to recognise that fungal fermentations are not limited to alcohol production by yeasts and other anaerobic processes. **Fermentation** refers to any of the biochemical transformations catalysed by fungi that are commercially significant and most of these processes are powered by aerobic metabolism.

THE CULTIVATION OF MUSHROOMS FOR FOOD AND PHARMACEUTICALS

Cultivation Methods

The global market for edible mushrooms has increased in recent decades and the marketplace has embraced an ever broader range of cultivated species. Most cultivated mushrooms are derived from natural varieties of wood-decay species of Basidiomycota. **Shiitake**, *Lentinula edodes*, is a white rot fungus that has been raised on logs for more than 1000 years. More than 1 million tonnes of shiitake are produced every year and China dominates the market. The **white button mushroom**, *Agaricus bisporus*, remains the most popular cultivated

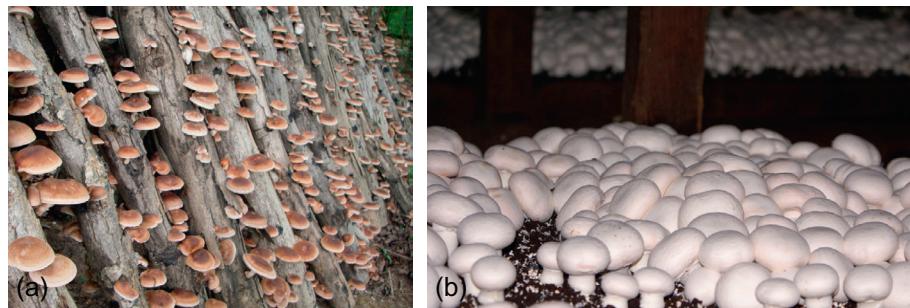


FIGURE 12.1 Cultivated mushrooms. (a) Shiitake, *Lentinula edodes*, on logs. (b) White button mushroom, *Agaricus bisporus*, on beds of compost. Source: panel (a) <http://www.sharondalefarm.com/cultivation/> and (b) <http://modernfarmer.com/2014/05/welcome-mushroom-country-population-nearly-half-u-s-mushrooms/>.

mushroom, with a global crop exceeding 2 million tonnes. Production of this mushroom in the United States approaches 400,000 tonnes and is valued at \$1 billion, but this is eclipsed by Chinese growers who process buttons for export. The methods of production for these market leaders offer a study in contrasts (Figure 12.1).

Mushroom cultivation begins with the production of a rich culture or spawn of the vegetative fungal mycelium, which is used as the inoculum. Shiitake spawn is raised on plugs of wood or on mixtures of sawdust, cereal bran, and other ingredients. Once the wood plugs or sawdust are colonised, the spawn is pressed into holes drilled into hardwood logs. The logs are kept in piles and covered with straw or with bags to maintain the high moisture levels that promote mycelial growth and wood decomposition. After 8–12 months, the logs are soaked with cold water to stimulate fruiting. Logs can produce multiple crops at intervals of a month or more for up to 6 years. Shiitake is also cultivated in plastic bags containing sawdust and a variety of waste agricultural products.

Agaricus bisporus spawn produced on cereal grains is used to inoculate beds of wheat or rice straw composted with animal dung and other ingredients. Before inoculation with the fungus, piles of these mixtures are fermented by thermophilic bacteria that change the nutrient content to favour the subsequent growth of the *Agaricus* mycelium. After up to 2 weeks of this cooking process, the compost is spread into wooden pallets and pasteurised by steaming to kill nematodes, insects, and other pests. The aim of steaming is to kill pests and competitors while minimising the loss of beneficial microorganisms. Fungi that can attack the mushroom mycelium and those that compete for nutrients are killed along with fungi that are potential pathogens of humans. Nitrifying bacteria that remove ammonia are unharmed by the pasteurisation process and residual ammonia is driven off by steaming. After pasteurisation, the compost is inoculated with spawn. Colonisation of the compost is stimulated by careful control of temperature, relative humidity, and carbon dioxide levels in the growing room. After 2–3 weeks, the compost is cased with a layer of peat and limestone in preparation for fruiting. The role of the casing is not known, but it is essential for fruiting. Peat is low in nutrients and the fungus produces short cords in the casing before mushroom primordia develop.

Fruiting is stimulated by reducing the temperature in the growing room from 25 to 18°C and increasing air circulation. In earlier stages of cultivation, the CO₂ concentration in the

growing room may be set as high as 3000 ppm. Airing reduces the levels of CO₂ and volatile organic compounds. Growers used to think that CO₂ served a major regulatory role in the initiation of fruiting, but recent studies show that a decrease in the concentration of volatile compounds (particularly 1-octen-3-ol, known as mushroom alcohol) above the mushrooms is the primary switch. Fruiting begins in 3 weeks and up to three flushes of mushrooms can be picked from a single bed. Although many of the details of production have evolved, the fundamental practice of cultivating mushrooms on a compost of animal dung has not changed since the technique was developed 300 years ago in France.

Mushrooms as Food and Medicine

Mushrooms have about the same calorific value as lettuce: more than 90% of the fresh weight of a mushroom is water and the remaining 10% is split between protein and carbohydrate in the form of insoluble fibre. The fat content of mushrooms is very low and the mixture of vitamins and minerals is unremarkable. Much of the culinary value of cultivated mushrooms derives from their flavour and the aroma of wild species is a determinant of their pricing. The most important volatile flavourings of mushrooms are C₈-derivatives, along with a variety of terpenoids and sulphur-containing compounds. The flavour profiles of mushrooms are exceedingly complex. More than 150 different volatiles have been identified in *Agaricus bisporus* using GC/MS and other analytical methods. The 'mushroomy' smell of the common edible species is produced by a mixture of compounds dominated by 1-octen-3-ol, which is also synthesised by fruits and potatoes. Other aromatic compounds in *Agaricus bisporus* include benzyl alcohol, benzaldehyde and cyclo-octenol. In some mushrooms, the level of benzaldehyde is higher than the C₈ compounds. Polyunsaturated fatty acids are the precursors for the synthesis of C₈ compounds. The lipid composition of mycelia and fruit bodies is very similar, but volatile production varies considerably between the caps and stalks of mushrooms. The natural functions of the volatile flavourings of mushrooms are unclear. In light of the importance of volatile levels in mushroom cultivation, it is possible that mushrooms, or clusters of mushrooms, use these compounds to suppress the fruiting of neighbours (conserving water and nutrients for their own mycelium). Another possibility is that the volatiles attract animals that serve as vectors for spore dispersal. This mechanism is crucial for the dispersal of truffles and many other fungi (Chapter 3). For mushrooms whose spores are spread by wind this seems paradoxical, but insects may play an accessory role in dispersal in some instances.

There are many claims about the medicinal properties of mushrooms. Mushrooms have been used in traditional medicines in China for centuries and there is a growing market for medicinal mushrooms outside Asia. Shiitake is the best-known medicinal mushroom and its dried basidiomata are used to treat a variety of ailments. Among its purported health benefits are its anti-tumour properties, anti-viral efficacy, and its utility in lowering serum cholesterol levels. Stimulation of the immune system is another of the therapeutic properties ascribed to shiitake. The majority of the claims about the health benefits of fungi are anecdotal, but shiitake has been the subject of a vast body of research and the mushroom's pharmacological properties have been linked to specific compounds in the fruit body. **Lentinan** is a water-soluble **beta-glucan** in the cell wall that appears to enhance the activity of dendritic cells that are involved in the recognition of cancer cells. Other studies have suggested that by stimulating an inflammatory response, lentinan acts as a powerful

anti-viral agent. Studies on a cell wall **proteoglycan** called polysaccharide K, from *Trametes versicolor*, show that this operates as an antioxidant and may inhibit tumour development in mice. Other mushrooms, including *Polyporus umbellatus* and *Hericium erinaceus*, have a broad range of medicinal uses and are recommended for the treatment of cirrhosis, hepatitis B, gastric ulcers, oesophageal cancer, and, like every other medicinal mushroom, are supposed to stimulate an impaired immune system. The majority of experiments that support these claims have been performed on tissue-cultured cells and mice. There are very few placebo-controlled double-blind clinical trials on human patients that demonstrate any benefit from treatment with medicinal mushrooms. Nevertheless, the business of medicinal mushrooms is booming.

Uses of Lichens

Lichens have been used as a source of dyes for centuries. They provided the reddish-brown, purple, and orange colours in Harris Tweed until synthetic dyes were adopted by Scottish manufacturers of this superb wool fabric. Lichens have also been used as food and in traditional medicine, and served as embalming agents in ancient Egypt. *Evernia prunastri*, or oak-moss, is a lichen that provides musky and woody scents in iconic fragrances formulated by French perfume houses. Compounds in extracts from this lichen can cause skin allergies and regulations have been introduced to reduce their concentration in perfumes.

PRODUCTION OF FOOD AND DRINK USING YEASTS AND FILAMENTOUS FUNGI

Wine and Beer

When oxygen is available, yeasts metabolise sugars to form carbon dioxide and water. If oxygen is scarce or absent, or if the sugar concentration is very high, metabolism follows a different fermentative pathway with the production of carbon dioxide and ethanol. This is the basis of the great variety of alcoholic drinks consumed by humans. Species of the ascomycete yeast, *Saccharomyces*, are responsible for the majority of alcoholic fermentations: *Saccharomyces cerevisiae* is used for fermenting beer and wine ([Figure 12.2](#)); lager production involves *Saccharomyces pastorianus*, and *Saccharomyces bayanus* is used for cidermaking and for producing Champagne and other sparkling wines. The association of these fungi with different beverages is complicated by the genetic diversity within single species of *Saccharomyces* and frequent errors in identification. *Saccharomyces pastorianus* (also known as *Saccharomyces carlsbergensis*) and *S. bayanus* are the products of complex hybridization events between other *Saccharomyces* species.

Wine and cider are made by fermenting plant juices rich in sugar. Beer is produced from starch-rich plant materials and the starch must be converted to sugars before fermentation. Drinks containing 10–12% alcohol are produced readily by natural fermentation. Higher concentrations of alcohol inhibit the metabolic activity of yeasts and the production of these more potent beverages involves distillation processes to boost the alcohol content after the initial fermentation. Spirits from sugary plant products include brandy (from grape juice), rum (from sugarcane), tequila (from agave), a variety of fruit brandies, and distilled versions of palm wines.

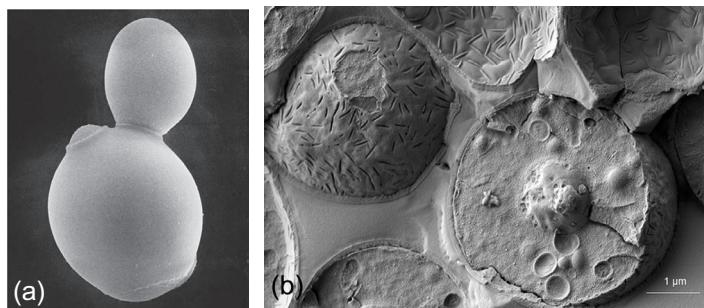


FIGURE 12.2 Electron micrographs of baker's yeast, *Saccharomyces cerevisiae*. (a) Scanning electron micrograph of mother cell with bud scar on upper left cell surface and less prominent birth scar at the bottom of the cell. Daughter cell at top right is approaching time of separation. (b) Transmission electron micrograph of freeze fractured yeasts showing outer surface of the cell wall (left) and its impression (top), and interior of whole cell containing prominent nucleus and nucleolus, and vesicles in the granular cytoplasm. *Source: Creative Commons.*

Spirits made from starchy materials include Scotch malt whiskey (from barley malt), gin (from barley, maize [corn], or rye), and vodka from various starchy sources including potatoes.

Wine and Other Beverages Produced from Sugary Juices

Red wines are fermented from grapes with red or purple/black skins whose phenolic pigments colour the wine (Figure 12.3). Grape skins and seeds are included in the first phase of fermentation of red wines. White wines are made from juice expressed from grapes without using the skins and seeds. Most white wines are produced from white grapes, but dark grapes are used for a few white wines because the pigments separate with the skin. After harvesting, grapes are crushed to break their skins and release the juice. True rosé wines are produced by including the grape skins at the beginning of the fermentation and discarding them after they have imparted the desired colour. This is called the skin contact method. The flavour of some white wines, including Sauternes, is enhanced by the colonisation of ripened grapes by *Botrytis cinerea* before picking. The fungus can destroy grapes under wet conditions, but its growth on drying grapes is called noble rot and produces a concentrated sweet white wine.

Wine fermentation is carried out in barrels, open vats, or industrial fermenters. Traditional wine production is carried out by the proliferation of yeasts that occur naturally on grapes. *Saccharomyces cerevisiae* is one of many grape yeasts and very little is known about its ecology and dispersal. It becomes the dominant species once the fermentation proceeds because it has quite a high tolerance to alcohol. Modern wine production usually involves the inoculation of the grape juice with a specific yeast strain. The juice is acidic (pH 2.9–3.9) which discourages the growth of bacteria and fungi that might otherwise spoil the fermentation. The addition of sulphur dioxide is an effective method for inhibiting the development of these undesirable microorganisms during the fermentation. *Saccharomyces cerevisiae* tolerates sulphur treatment and continues to grow until the alcohol concentration reaches 10–12%. Once this threshold alcohol level is reached, yeast metabolism slows and cell numbers decline. The remaining concentration of unfermented sugars determines the level of sweetness of the wine. Yeast metabolism during the fermentation generates hundreds of compounds that affect wine flavour. Glycerol adds 'body' to the wine and volatile ethyl esters are important in determining aroma.



FIGURE 12.3 Modern winery with high capacity stainless steel fermenters. Source: Fedor Kondratenko©123RF.com

Following the first phase of the fermentation, the wine is transferred to wooden casks and other containers for maturation and storage. Lactic acid bacteria carry out malolactic fermentation, converting strongly acidic malic acid to blander lactic acid and raising the pH. The wine is filtered before bottling to eliminate bacteria that might spoil the wine by converting the ethanol to acetic acid and carrying out other reactions.

Cider apples are classified according to sweetness, ranging from bittersharp (high in acidity and tannin) to sweet (low acidity and low tannin). The apples are crushed and the pulp is pressed to release the juice. Traditional cidermaking relies upon yeasts carried on the apple skin, but, like winemaking, modern cidermaking involves inoculation with specific yeast strains and addition of sulphur dioxide to exclude other microorganisms. Apple juice has lower sugar content than grape juice, which results in a beverage with a lower alcohol content than wine. Following alcoholic fermentation, lactic acid bacteria may reduce the acidity of the fermentation. Bottled cider is pasteurised or filtered to prevent spoilage. Perry is produced from pears using similar methods.

Wines are made from many kinds of fruit using the same methods used to ferment grape juice, and palm wine made from the sugary sap of palm trees is popular in the tropics. Palm trees are tapped and their sap is fed into gourds or other containers. Rapid fermentation is catalysed by natural yeasts and the wine is consumed swiftly before bacterial spoilage occurs. Pulque is a Mexican wine produced from agave sap.

Beer Production from Starchy Materials

Beers made from a variety of grains and root crops rich in starch have a huge global market (Figure 12.4). European style beer is an alcoholic beverage made from malted barley and flavoured with hops. The German beer purity law, or Reinheitsgebot, dating to the sixteenth century, limits beer ingredients to malted barley, water, hops, and yeast. Brewers with a less stringent attitude to beer-making add other kinds of cereal, fruit juices, and diverse



FIGURE 12.4 Open vat fermentation to produce wheat beer. Rolling fermentation in centre of the tank is caused by carbon dioxide bubbling up from submerged yeasts. Source: <http://brewercameron.wordpress.com/2012/06/28/bavaria-excursion-day-3/>.

chemicals to create different flavours, control foaming, and control other characteristics. Ales and wheat beers (German weissbiers) are produced with **top yeasts** and served at room temperature or slightly cooled; lager is produced by a **bottom yeast** and is consumed after refrigeration. Top yeasts mix with the gas that accumulates as foam at the surface of the traditional open vat. Bottom yeasts tend to sediment to the bottom of the vat. These fundamental differences in behaviour are unimportant when a closed fermenter is used for brewing.

Few yeasts can utilise starch, so the conversion of starch into sugars, or **saccharification**, must be carried out before fermentation can commence. In beer brewing, saccharification is known as **malting**. Barley grains are steeped in water for up to 24h and allowed to germinate under moist aerobic conditions. During germination, proteinases and amylases break down proteins and starch into amino acids and sugars. After 1 week, the germinating grains are dried with a stream of air heated in a kiln. The dried grains are known as **malt**. If the **kilning** process is carried out at 60–79 °C, the embryos in the grain are killed but the enzymes retain their activity. This active malt is used to produce light-coloured beers. Kilning at higher temperatures destroys the enzymes and this kind of malt is added to the more active type to produce darker beers.

After kilning, the malt is milled into a coarse flour or grist. This is added to warm water to produce a mash in which enzyme activity continues to modify the proteins and starch. In beers that ignore the German purity standard, corn or rice grits are added to increase the starch content of the mash. After a few hours, the solids are separated from the mash to leave a sugar-rich liquid wort. Syrups are added to increase the sugar content of some beer worts. In the next step, dried female flowers of the hop plant, *Humulus lupulus*, are added to the wort and the mixture is boiled. Boiling denatures the enzymes, coagulates some of the proteins, and sterilises the wort. Cyclic organic compounds in the hops, called humulones, isomerise to form isohumulones that make the beer bitter, and other molecules impart different flavours and aromas. After the wort is cooled, hop residues and other solids are removed, and the wort is inoculated with a specific yeast strain. The wort is aerated to promote yeast growth and

fermentation proceeds in open tanks or in closed stainless steel fermenters. Cylindro-conical fermenters are the most popular designs used in today's breweries, in which circulation of the contents is driven by carbon dioxide bubbles produced by the fermentation, or forced into the tank via a sparger. The carbon dioxide bubbles rise to the surface through the centre of the tank, and cooler liquid descends on the outside toward the conical base.

Temperatures for ale fermentation (15–25 °C) are higher than those used for lager (8–15 °C). Oxygen levels decline very quickly during the fermentation and most of the process is anaerobic with efficient conversion of sugar to alcohol rather than biomass. The yeasts produce a variety of compounds that flavour the beer. Yeasts which are particularly good for brewing flocculate and clump toward the end of the fermentation, which facilitates their removal. Maturation in casks follows and a secondary fermentation of residual sugars is completed by low concentrations of yeast remaining in the beer. The final step of clarification of the beer, or fining, involves chemical treatment to cause sedimentation of particles (isinglass prepared from the dried swimbladders of fish is the traditional additive) followed by filtration. Pasteurisation reduces opportunities for spoilage.

Beer with up to 0.5% alcohol, marketed as alcohol-free beer, is made by adapting the mashing process to limit **saccharification** and by reducing the wort gravity (a measure of its sugar content) at the beginning of the fermentation. Yeast strains with limited fermentative performance are also utilised by some brewers and alcohol content can be reduced by vacuum distillation or reverse osmosis once the brewing process is complete.

Baking

Saccharomyces cerevisiae has been used to ferment dough made from wheat and rye flour for more than a millennium. Top yeast harvested as a waste from beer brewing was used in bread making by Romans and this practice was widespread in the nineteenth century. Baker's yeast is manufactured today using fed-batch fermentation with molasses supplemented with a variety of nutrients (p. 413). Fed-batch fermentation allows the operator to add nutrients to the reaction continuously or intermittently to control the metabolic activity of the cells and generate high cell densities. The yeast cultures are aerated to maximise respiration and biomass accumulation and conditions are regulated to stimulate trehalose synthesis. *Saccharomyces* produces the sugar alcohol trehalose as a storage carbohydrate and it protects the cells when they are freeze-dried. The yeast is harvested by vacuum filtration and prepared for baking as freeze-dried granules, compressed cakes, or in a concentrated liquid form called cream yeast. Cream yeast has a limited shelf life, but is preferred by large bakeries. Dough is prepared by mixing flour with water, yeast (2%) and salt (1.5–2%). Amylases in the dough break down the starch and form glucose, maltose (disaccharide of glucose units), and maltotriose (trisaccharide of glucose), which are fermented by the yeast. Maltose is the dominant sugar. Milk, sugar, eggs, and other ingredients are added to the dough to produce different kinds of bread and mixing (kneading) of the dough ensures uniform distribution of the ingredients. As the dough is kneaded, proteins in the flour, called gliadin and glutenin, form strands of gluten that give the dough its springy and elastic texture and are responsible for the chewiness of the baked bread. After kneading, the dough is left for a few hours and the yeast ferments the sugars and releases carbon dioxide that causes the dough to rise. Ethanol produced during this fermentation kills the yeast and is expelled during baking.

Cheeses and Meat Products

Lactic acid bacteria ferment lactose in milk to lactic acid and produce many of the metabolites that impart characteristic flavours to cheeses. The enzyme **chymosin** (also known as rennin) is added to coagulate the milk and form curd. Traditionally, this has been obtained from the stomach lining of unweaned calves. Most of commercial cheese production today relies on **fermentation-produced chymosin** (FPC) from recombinant strains of *E. coli* and the ascomycetes *Aspergillus niger* var. *awarmori* and *Kluyveromyces lactis* (a yeast). Natural proteases obtained from species of *Rhizomucor* offer a 'vegetarian' alternative to the use of these recombinant microorganisms. Yeasts and filamentous fungi also play subsidiary roles in flavouring and ripening cheeses. Brie and Camembert are well known surface-ripened cheeses whose rinds are formed from a dense white mycelium of *Penicillium camemberti* (Figure 12.5). These cheeses are salted during preparation and the halotolerance of the fungus allows it to grow on the surface. Enzymes secreted from the rind penetrate the outermost millimetres of the cheese and these proteinases and lipases add fruitiness and fragrance. Like these soft cheeses, Italian salami is preserved and flavoured by a thinner coating of *Penicillium nalgiovense* and other *Penicillium* species. *Penicillium roqueforti* is the filamentous fungus that proliferates within the blue-vein cheeses including Roquefort, Gorgonzola, Stilton, and Danish Blue. The bacteria that ferment the milk for these varieties produce carbon dioxide that creates irregular cavities in the cheese. *Penicillium* conidia are included in the starter culture or added to the fresh curd. After the curd is compressed, salt is dusted on the surface and diffuses into the cheese. The cheese is spiked after salting and the resulting aeration allows the conidia of the fungus to germinate and colonise the cavities. The blue coloration of the cavities is caused by sporulation, and enzymes secreted by the fungus impart glorious flavours to the mature cheese.

Yeasts are involved in fermentation reactions and maturation processes in cheese production, but many of their effects on flavour and texture are unclear. *Debaryomyces hansenii* grows in semi-soft cheeses and is important in surface ripening of Munster, Limburger, and Port Salut; *Geotrichum candidum* is added to soft cheeses; *Yarrowia lipolytica*, *Saccharomyces cerevisiae*, and *Kluyveromyces* species are also common ascomycete yeasts in cheeses.



FIGURE 12.5 Camembert cheese with characteristic white rind of *Penicillium camemberti* mycelium. Source: <http://www.pnwcheese.com/2011/07/kurtwood-farms-dinahs-cheese-coming-soon-to-portland.html>

Chocolate

Cacao seeds, or 'beans', are embedded in white pulp inside the colourful pods of *Theobroma cacao* trees. To manufacture chocolate, the pods are cut open, the pulp-bean mass is arranged in heaps, boxes, or baskets and allowed to ferment in the open air for 4–7 days. Fermentation of sugars and citric acid in the mucilaginous pulp kills the cacao embryos and reduces the astringency of the seeds. Cacao fermentation involves a complex community of yeasts and bacteria. *Hanseniaspora guilliermondii* and *Hanseniaspora opuntiae* dominate the initial stages of the fermentation and are followed by multiple strains of *Saccharomyces cerevisiae*, *Pichia kudriavzevii*, and other yeasts. The succession of microorganisms reflects changes in the physical and chemical environment within the pulp-bean mass. Bacteria that ferment the citric acid in the pulp create the conditions that favour the growth of *Saccharomyces cerevisiae* and this species outcompetes yeasts that are less tolerant of the increase in temperature and ethanol concentration in later stages of the fermentation. At the end of the fermentation, the seeds are dried, their thin husks are removed, and the remaining tissue is ground to produce nibs. The nibs are combined with other ingredients to produce chocolate.

Asian Fermented Foods

The greatest variety of foods and beverages produced by fungal fermentation are found in Asia. **Tempe**, originating in Java, is produced by fermenting beans or cereals and is a popular meat substitute. The traditional production method begins with the de-hulling of soybeans, followed by soaking and boiling in water before the beans are spread on trays to allow rapid cooling and evaporation of water. After cooling, the beans are inoculated with spores of *Rhizopus* or *Mucor* species (Mucorales), mixed, spread into layered beds, and incubated for 1–2 days allowing the development of a dense mycelium. The mycelium transforms the raw soybeans into a firm, sliceable cake and its enzymes modify the polysaccharide content of the beans and improve the digestibility of tempe. Other biochemical transformations by the fungus include the generation of antioxidants with purported health benefits. Different varieties of *Rhizopus microsporus* (another mucoralean) are the most common fungi used for manufacturing tempe. These grow in soil and on leaves and are prevalent in the air spora. Rather than hoping for natural colonisation with the correct fermenters, tempe is inoculated with a **starter** enriched in the fungus. Starters are used to introduce fungi into the manufacture of a variety of Asian foods. They are made from rice or wheat dough flavoured with spices. The dough is shaped into small biscuits, inoculated with dry starter from an earlier batch, and incubated for up to 5 days. After drying in the sun, the new starters can be stored for several months before they are used to produce tempe, soy sauce, miso, saké, sweetened rice, and other foods. Some starters contain pure cultures of a particular fungus, others comprise a variety of yeasts and filamentous fungi.

Furu or **Sufu** is a cheese-like food produced from soybeans in China. It is manufactured according to a complicated three-stage process that begins with the extraction of soy milk from the beans to form a curd. The curd is pressed into tofu blocks which are heated, inoculated with a starter containing an *Actinomucor*, *Mucor*, or *Rhizopus* species, and fermented for up to 1 week. In the third stage, the fermented tofu is ripened in jars of brine for 2–4 months. **Soy sauce** production is similarly complex. Soybeans are soaked in water, boiled, drained, mixed with roasted wheat and spread on trays, inoculated with *Aspergillus oryzae* or *Aspergillus sojae* and fermented for 5 days to form koji. The koji is mixed with brine to produce moromi which is fermented by yeast and lactic acid bacteria for 1 year. The filamentous fungi are destroyed

during this second stage fermentation, but their enzymes remain active in the brine. Other Asian fermented foods include red koji rice, fermented by *Monoascus*, saké and other rice wines, and Chinese liquor or jiu made from sorghum.

FERMENTATION TECHNOLOGY

Fermentation technology is concerned with the large-scale culture of microorganisms in fermenters, and the recovery of useful products from the metabolic activity of the microbial cells. Industrial mycologists study the fungi used to perform chemical transformations of commercial significance, the food sources (feedstocks) on which the fungi are cultured, optimization of fermenter design and operation, and 'downstream' processing to harvest the desired products.

Feedstocks

For commercial success, a fungus must behave in a consistent, predictable fashion, showing similar growth rates and metabolite yields in successive fermentations. The provision of specific nutrients is crucial in controlling the growth and development of the fungus. In the laboratory, this can be achieved by producing a defined medium by mixing pure chemicals in defined concentrations. This is not feasible for a large-scale industrial process and the selection of raw materials for a particular fermentation is based on nutrient content, cost, and availability. Downstream processing costs also vary according to the feedstock. Purification of the product from the partly digested raw materials can be expensive, and waste disposal represents an additional cost and can pose environmental hazards. Glucose and sucrose are excellent carbon sources for most fungi so most feedstocks include sugarcane juice, unrefined sugar, molasses, or hydrolysed starch. Vegetable oils are useful alternative feedstocks for some fermentations. Some feedstocks provide the fungus with a combined carbon and nitrogen source, but a separate nitrogen source is supplied to many processes. In some cases, vitamins are added as yeast extract, as well as additional minerals and trace elements. The cost of the raw materials for the fermentation ranges widely, representing more than 50% of the production budget for industrial ethanol.

Fermenter Design and Operation

Most industrial fermentations are carried out in **stirred tank fermenters** in which sterile liquid medium can be inoculated, aerated, stirred, monitored by sensing instruments, heated or cooled, and sampled or fed with additional materials without introducing contaminants ([Figure 12.6](#)). Fermentation tanks or **bioreactors** are manufactured from stainless steel to avoid corrosion and leaching of toxic metals into the medium. Industrial fermentations employ **batch culture** and **continuous culture** methods. In batch culture, the level of nutrients declines as the density of cells increases and the fermentation is stopped to harvest the product. Continuous culture allows the operator to maintain optimal conditions for fermentation for many weeks by programming cycles of nutrient injection and other changes in the fermentation conditions. Fermentation products can be harvested repeatedly or continuously using this method. Because many fermentation products are secondary metabolites, they are formed when the growth rate of the fungus begins to stall. Batch cultures are preferred for these kinds of fermentations because the classic growth kinetics characterised by lag,

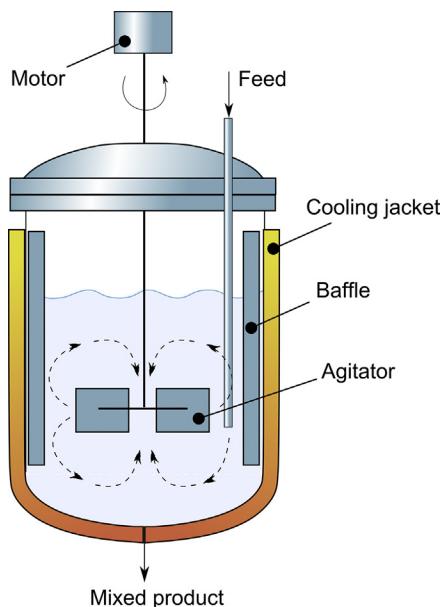


FIGURE 12.6 Stirred tank fermenter or bioreactor. *Source: Creative Commons.*

exponential, and stationary phases occur without manipulating the growth conditions. The same results can be obtained via continuous culture by programming successive cycles of nutrient injection, followed by starvation and secondary metabolite synthesis. This is, however, unnecessarily complicated and expensive when batch culture works so well. Continuous culture is recommended for other processes in which large quantities of a product associated with growth are needed. Examples of continuous culture include the production of ethanol and single cell protein (mycoprotein). **Fed-batch culture** is a hybrid of these methods in which nutrients are added to the culture during the fermentation. This avoids the problems associated with catabolite repression (Chapter 5) in which high levels of sugars added at the start of conventional batch culture inhibit secondary metabolism. Fed-batch culture is not the same as continuous culture, because the fermentation is terminated when the product is harvested. Fed batch culture is used to produce antibiotics (pp. 418–419).

Heat sterilization of the fermenter and growth medium before inoculation is critical. This can be done inside the fermentation tank by heating the surrounding jacket and injecting steam into the reactor. The gas exit valve must be closed to allow pressurization and heating of the system above 100 °C. Alternatively, medium can be sterilised outside the fermenter and supplied via sterile lines. Air for aerobic fermentations is pumped into the medium through a sparger to generate air bubbles that facilitate oxygen diffusion throughout the reactor volume. Agitation of the medium with a stirrer enhances this process.

Downstream Processing

One litre of broth may contain 1 g of an enzyme product or a few grams of antibiotic, which means that a small quantity of product must be separated from a large volume of waste

material. Fungal mycelium is separated from broth using a rotary vacuum filter. Yeast cells are removed by flotation and sedimentation in brewing practices and centrifugation has been introduced to standardise many modern biotechnological processes. In fermentations where intact cells are harvested to collect intracellular enzymes, water is removed from the culture and enzymes are recovered by breaking the cells using a variety of methods. Processing of antibiotics and many other products dissolved in the fluid phase of the fermentation involves harvesting the broth and treating the fungal biomass as solid waste.

Solid-State Fermentations

Traditional production methods for Asian foods (see above) are solid state fermentations (also known as **solid-substrate fermentations**) in which fungi are grown on soybeans and cereal grains. The raw materials are treated prior to inoculation to facilitate subsequent transformation by the fungus. These pre-treatments include grinding or milling to increase surface area, soaking to hydrate and soften the food base, and steaming to kill the seeds and eliminate other microorganisms. Because the fungi used in these processes are aerobes, moisture content must be kept quite low during some fermentations to maintain air spaces in the nutrient source. Asian foods are produced on stacked wooden trays that provide aeration and large surface areas for fungal growth. Adaptation of these sorts of methods for industrial fermenters is complicated, but the use of a forced air supply combined with rotating drums can maintain adequate aeration. Mushroom cultivation and composting of agricultural wastes are other examples of solid-state fermentations.

GENETIC MANIPULATION OF FUNGI FOR BIOTECHNOLOGY

The most important filamentous fungi used for protein production are *Aspergillus niger*, *Aspergillus oryzae*, and *Trichoderma reesei*. Beyond the selection of naturally occurring strains of these species that show high levels of secretion of the protein of interest, random mutagenesis can be used to isolate mutants with enhanced synthetic and secretory performance. Genetic engineering of wild type or mutant strains to boost protein production has also proven very effective and dominates modern biotechnological research. Proteins that are natural products of a particular fungus are called **homologous proteins**. A fungus can also be engineered to produce foreign or **heterologous proteins**. Production of heterologous proteins is dependent upon the genetic alteration of a fungus through the incorporation of exogenous genes. This is called **transformation**. Several methods are used to transform *Aspergillus* and other filamentous fungi with genes carried on **plasmids**. Plasmids are small DNA molecules that replicate independently from chromosomal DNA. The plasmids used for transforming fungi originated in bacteria. Natural plasmids are also found in fungal mitochondria, consistent with the bacterial origin of these organelles, and multiple copies of the 'yeast 2-micron plasmid' are found in the nucleus of *Saccharomyces cerevisiae*.

The fungal cell wall is a macromolecular sieve that presents a barrier to plasmid uptake. This is circumvented by digesting the cell wall of germinating conidia with a cocktail of enzymes. The resulting protoplasts absorb plasmids when they are incubated in a solution containing calcium chloride and polyethylene glycol. Other methods for plasmid transfer include **electroporation** and use of the bacterium *Agrobacterium tumefaciens* as a live vector.

Successful transformation requires the integration of the foreign DNA into homologous or non-homologous regions of the host genome. Integration of the heterologous gene can be targeted to a specific locus in the host genome and genetic engineers favour regions of the genome that encode secreted proteins that are already expressed at high levels. If this is done by replacing the native gene, expression of the heterologous gene may be further enhanced by the lack of competition for secretory machinery from the native gene product. Increased production of homologous and heterologous proteins has been achieved by introducing **multiple copies** of a gene and driving their transcription with the use of a **strong promoter**. The *gpdA* promoter from *Aspergillus nidulans* is part of a gene that encodes the glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase. This constitutive promoter is valuable in basic research and biotechnological applications because it functions in other fungi (Figure 12.7).

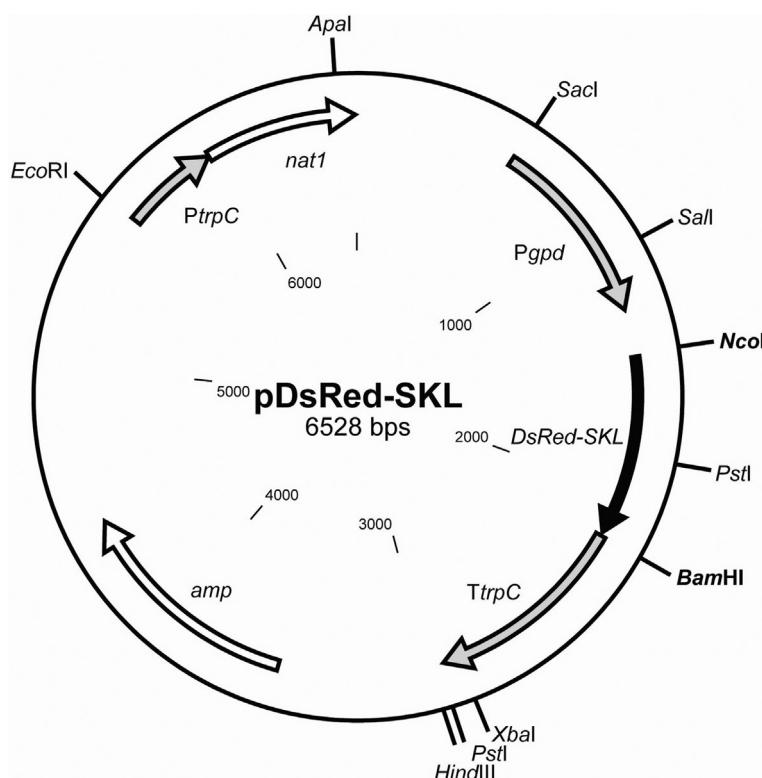


FIGURE 12.7 Example of plasmid construct used in a cell biological study of the ascomycete *Sordaria macrospora*. The DsRed-SKL plasmid incorporates a gene derived from a jellyfish (*DsRed*) that encodes a red fluorescent protein. This gene is fused to the sequence for a trio of amino acids (serine-lysine-leucine, or SKL) to produce a protein that is incorporated into peroxisomes (organelles involved in fatty acid breakdown). Transcription of *DsRed-SKL* is driven by a constitutive promoter (*Pgpd*) derived from *Aspergillus nidulans*. *TtrpC* is a terminator sequence, derived from *Escherichia coli*, which stops transcription and releases the mRNA that is translated into the DsRed-SKL protein. The positions of restriction sites are indicated with the labels on the outside of the plasmid. Expression of this plasmid in *Sordaria* allowed investigators to study the cellular distribution of peroxisomes using fluorescence microscopy. Source: Elleuche, M., Pöggeler, S., 2008. *Fungal Genet. Rep.* 55, 9–12.

Inducible promoters are used in many applications and these include promoters of genes that encode numerous secreted enzymes. Homologous promoters tend to work better than heterologous promoters.

Several heterologous proteins have been produced in *Trichoderma reesei* at levels of commercial significance including a phytase from *Aspergillus* (2 g/L), glucoamylase from the creosote fungus, *Hormoconis resinae* (0.7 g/L), and xylanase from the thermophilic soil fungus, *Humicola grisea* (0.5 g/L). Comparable yields of human immunoglobulins and interleukin 6 have been produced by recombinant strains of *Aspergillus niger*. An engineered strain of *Aspergillus oryzae* is a source of a heat-stable lipase encoded by a gene from a thermophilic ascomycete, *Thermomyces lanuginosa*. This enzyme is useful as an additive to laundry detergents and has been modified via a single amino acid substitution to be effective at low wash temperatures.

MODERN BIOTECHNOLOGICAL APPLICATIONS OF FILAMENTOUS FUNGI AND YEASTS

Single Cell Protein

The term single cell protein was introduced in the 1960s to describe protein-rich foods manufactured from yeasts that served as dietary supplements for livestock and humans. Single cell protein was viewed as a product category that might address food shortages at a time when it seemed unlikely that agricultural production could keep pace with the skyrocketing human population. Interest in food yeast declined as improvements in plant breeding and agricultural practices led to the contemporary boom in global food production. *Saccharomyces cerevisiae* produced in stirred fermenters on molasses is an example of single cell protein that is manufactured today. The yeast produced in this fashion is not consumed directly, but is used for baking. Marmite is a savoury spread made from yeast extract that has been popular in the United Kingdom for more than a century. Spent yeast from beer brewing is used to produce this sticky dark brown paste. Vegemite is a similar product made in Australia.

Another fungal protein product, called **Quorn**, is a very successful meat substitute manufactured by a single strain of a filamentous saprotrophic ascomycete, *Fusarium venenatum*. Because Quorn is produced from a multi-cellular, filamentous fungus, the term single cell protein is inaccurate and **mycoprotein** is the preferred name. The use of a filamentous fungus makes it possible to produce a meat-like consistency that cannot be replicated with a single-cell protein. The fungus is grown in pairs of 50 m tall air-lift fermenter vessels that contain 230 tonnes of broth ([Figure 12.8](#)). The vessels are connected at the top and the bottom to form a continuous loop. Compressed air and ammonia are pumped into the bottom of the first vessel, called the 'riser', oxygenating the culture and circulating the liquid containing the fungus toward the top. Carbon dioxide from the respiring cells is released through a vent at the top of the system, and the liquid falls through the second 'downcomer' vessel and is infused with fresh nutrient solution (glucose plus vitamins and minerals). A heat exchanger at the bottom of the system maintains the temperature at 30 °C and the culture is harvested at a rate of 30 tonnes per hour. The dense mycelium of branched hyphae harvested from the fermenter has a very high RNA content. This is problematic for a food product because its consumption could raise uric acid levels in the blood and lead to gout and other illnesses. This is addressed by heating the mycelium

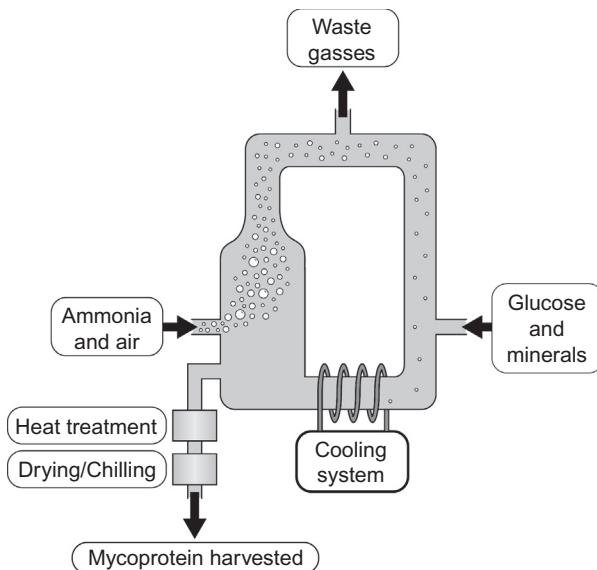


FIGURE 12.8 Air-lift fermentation system used to produce Quorn. *Mark Fischer, Mount St. Joseph University, Cincinnati*

at 68°C for 20 min, which allows endogenous enzymes to destroy much of the RNA without reducing its protein content. The heated mycoprotein is then dried and bound with egg white. Further processing creates the meaty texture and adds flavourings and colourings.

Fungal Enzymes

The secretion of enzymes that service the absorptive feeding mechanism of the fungi is a huge asset for biotechnology because fungi release many protein products into their culture fluid simplifying purification. A particular advantage of using fungi over bacteria for protein production is that they engage in eukaryotic post-translational processing of proteins which is essential for the biological activity of many heterologous proteins of pharmaceutical relevance. At the same time, fungi are easily cultured like bacteria. The global market for enzymes generated by fermentation exceeds US \$5 billion, and fermentation by filamentous fungi accounts for half of this production. Biotechnological applications of specific fungal enzymes impact a vast array of products. More than one hundred enzymes have been commercialized from 25 genera including the ascomycetes *Aspergillus*, *Trichoderma* and *Penicillium*, the zygomycete *Rhizopus*, and the basidiomycete *Humicola*. **Fungal hydrolases** are the most important class of enzymes with applications in the food and beverage industries, personal care products, laundry detergent manufacture, textiles, leather, forest products, animal feed, and biofuels. The use of live fungal cultures in baking, brewing, and the manufacture of other foods was discussed earlier in this chapter, but purified enzymes have other uses in food products. These include mixtures of enzymes that hydrolyse proteins in meat products and vegetables to produce soups, stock cubes, a variety of sauces, and the ubiquitous food flavouring monosodium glutamate. As mentioned earlier (p. 409), fungal enzymes that act

as coagulants are used in cheese production and a recombinant version of bovine chymosin produced by *Aspergillus niger* is a substitute for the enzyme obtained from calves. **Lactases** from *Aspergillus* and *Kluyveromyces* increase the digestibility of dairy products for consumers with lactose intolerance. Lactases are also effective at digesting antigens in milk to produce milk formulas for infants.

Fungal enzymes have been used in toothpastes, to combat bacteria active in tooth decay and enhance bleaching, and the addition of a **laccase** from the wood-decaying basidiomycete *Trametes* to breath fresheners destroys the compounds responsible for halitosis. Fungal proteases and amylases in laundry detergents degrade dirt particles, and lipases are active against grease stains. **Cellulases** in detergent formulations increase the softness and brightness of clothing. Enzymes from fungi and from bacteria allow washing at lower temperature, which reduces energy consumption, and water use is reduced by their effectiveness at removing dirt. Cellulases from *Trichoderma reesei* create the popular stonewashed appearance of denim. Processing of raw cotton involves bacterial and fungal enzymes; catalases from *Aspergillus* and other fungi are used to bleach cotton before it is dyed, and cellulases are used in the finishing process to increase the smoothness of the fabric and reduce 'pilling'.

In papermaking, fungal **xylanases** are used in chlorine-free bleaching to hydrolyse the hemicelluloses in wood pulp. This has the effect of opening the molecular structure of the pulp which releases the dark-coloured complexes of insoluble lignin. Lipases, amylases and cellulases are also used in paper manufacture and recycling. Fungal xylanases are vital in many other applications including the degumming of flax, jute and hemp, silage and grain processing, and as additives to wheat flour to improve dough handling. Fermentation of cereals with beta-glucanases from *Trichoderma* and *Aspergillus* increases their nutritional value for monogastric animals like pigs and poultry. Fungal **phosphatases (phytases)** that release phosphorus from polymeric stores are also used for processing animal feed.

Finally, fungal enzymes may play an increasingly significant role in the biofuel industry. After milling, whole grains can be cold-cooked with **glucoamylases** and **alpha-amylases** to produce fermentable sugars including glucose and maltose. Dry plant matter, or lignocelulosic biomass, is a huge untapped feedstuff for **bioethanol** whose utilisation will require new approaches for using fungal enzymes to release fermentable sugars from these complex polymers (pp. 421–422).

Organic Acid Synthesis

Aspergillus species are regarded as the most important industrial microorganisms. *Aspergillus niger* is a global source for the production of more than 1 million tonnes of **citric acid** per year, which is used as a preservative and chelating agent, and as an acidic flavouring for foods and soft drinks. *Aspergillus niger* and *Penicillium* species produce **gluconic acid** which is used for cleaning and finishing metal surfaces, and as an additive in cement. Lower concentrations of gluconic acid are also used as a food additive. Annual production is estimated at 50,000–100,000 tonnes. Gluconic acid is generated by a two-step oxidation of glucose by a pair of enzymes secreted by *Aspergillus niger*. **Itaconic acid**, used in polymer synthesis, is produced by *Aspergillus terreus* and *Aspergillus itaconicus* in a submerged fermentation of glucose syrup or molasses. Kojic acid is another *Aspergillus* product that is sold as a cosmetic for whitening skin. It is a byproduct of soy sauce fermentation.

Vitamin B₂ Synthesis

Industrial production of vitamin B₂, or riboflavin, involves the fermentation of soybean oil and soybean meal by the ascomycete *Ashbya gossypii*. Triglycerides in the broth are hydrolysed by a lipase secreted by the fungus. Free fatty acids are then absorbed by the fungus and used as precursors for riboflavin synthesis. Industrial strains of *Ashbya gossypii* have been produced by a combination of classical strain selection and molecular engineering of the riboflavin pathway to stimulate vitamin synthesis.

Antibiotics and Other Pharmacological Agents

β -Lactam Antibiotics

Penicillins, secreted by species of the ascomycete *Penicillium*, are powerful anti-bacterial agents that target Gram-positive bacteria. They are members of a class of antibiotics that share a core structure called the β -lactam ring (Figure 12.9). Other β -lactam antibiotics include **cephalosporins**, produced by the fungus *Acremonium*, and **carbapenems** produced by bacteria. The production of **penicillin G** (Gold Standard penicillin) by *Penicillium rubens* (identified originally as *Penicillium chrysogenum*) was discovered by Alexander Fleming in 1928 and developed for clinical use by a research team of Oxford University scientists led by Howard Florey. Research to increase production of the antibiotic began in 1938 and the drug was tested on the first patient in 1941. A strain of *Penicillium chrysogenum* isolated from a rotting cantaloupe in 1943 gave much higher yields of the antibiotic and this fungus became the mainstay of industrial production. Penicillins and other β -lactam antibiotics destroy bacterial cells by interfering with the formation of cross-links within their peptidoglycan cell wall. Peptidoglycan is a polymer formed by chains of alternating residues of a pair of amino sugars, *N*-acetylglucosamine and *N*-acetylmuramic acid, with short peptides attached to the *N*-acetylmuramic acid residues. Cross linkages between amino acids in different peptide chains creates a strong three-dimensional crystal lattice. The antibiotic binds to proteins called penicillin-binding proteins or transpeptidases that form the bonds between the cross-linking

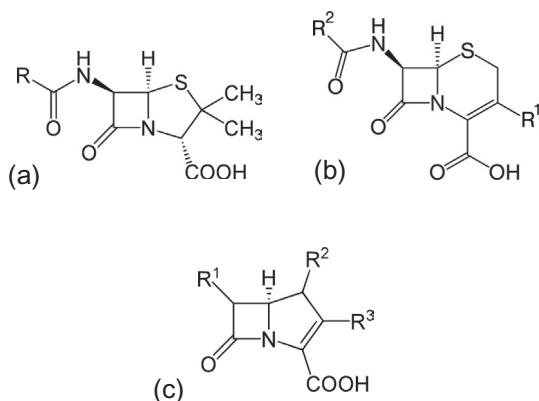


FIGURE 12.9 Structures of β -lactam antibiotic molecules. Core structures of (a) penicillins, (b) cephalosporins, (c) carbapenems. Source: Creative Commons

peptides in the wall. Inhibition of these enzymes weakens the cell wall and causes the bacteria to burst. Bacterial resistance to penicillin derives from the evolution of strains that produce enzymes called **β -lactamases** that inactivate the antibiotic by opening the β -lactam ring. Bacterial resistance became a problem within the first decade of penicillin's use. An important advance was made with the discovery that *Penicillium chrysogenum* produces large quantities of 6-aminopenicillanic acid (6-APA), which is the core structure of the antibiotic. This structure lacks antibiotic activity, but can be used to produce semi-synthetic antibiotics by adding a variety of side chains. **Meticillin** (formerly methicillin) and **ampicillin** are examples of these compounds. Meticillin is resistant to β -lactamase due to the steric hindrance offered by its bulky side chain that blocks access to the β -lactam ring. Bacterial resistance to meticillin has led to its replacement with other antibiotics. Ampicillin is a broad-spectrum antibiotic that is active against Gram-negative as well as Gram-positive bacteria.

Cephalosporins are β -lactam antibiotics produced by *Acremonium chrysogenum*. The first cephalosporins, called first generation cephalosporins, were active against Gram-positive bacteria. Later generations of these antibiotics, produced by cleaving the parent molecules and adding various side chains, have shown greater efficacy against Gram-negative bacteria, sometimes at the expense of reduced potency against Gram-positive bacteria. These are exceedingly valuable antibiotics, with the fifth-generation cephalosporins representing the last line of defence against infections caused by meticillin-resistant *Staphylococcus aureus* (MRSA).

Lovastatin

Lovastatin is a fungal polyketide employed as a cholesterol-lowering drug that inhibits (3S)-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase. This enzyme catalyses the synthesis of mevalonate, which is the immediate precursor of cholesterol and is the target of all of the drugs classed as statins. Lovastatin was discovered in *Aspergillus terreus* and *Monascus ruber* in the 1970s and is a natural product in oyster mushrooms (*Pleurotus ostreatus*) and red yeast rice (rice fermented by *Monascus*). It is produced by a multi-domain enzyme complex known as a **polyketide megasynthase** encoded by a cluster of genes including *lovA*, *lovB*, *lovC*, *lovD*, and *lovF*. The biosynthetic pathway resembles the pathway of fatty acid synthesis where the polymer is extended with the repetitive addition of acetyl units. Subunits of the enzyme complex are organised in modules that initiate the synthesis (starter module), add acetyl units from malonyl CoA to the growing polyketide chain (elongation module), and terminate the reaction (termination module). Lovastatin is prescribed under the name Mevacor, and a synthetic derivative, called simvastatin, is trademarked as Zocor. These drugs, together with fully-synthetic statins including Lipitor, produce multibillion-dollar sales for Pfizer, AstraZeneca, Merck, and Novartis.

Immunosuppressants

Cyclosporin A is an immunosuppressive, fat-soluble, cyclic peptide that blocks the activation and proliferation of CD4+ and CD8+ T lymphocytes (T helper cells and CD8 killer cells, respectively) by inhibiting the production of the cytokine, interleukin-2 (IL-2). In addition to its effectiveness in supporting patients after bone marrow and organ transplantation, cyclosporin A is used to treat a wide range of illnesses including psoriasis, severe atopic dermatitis, and rheumatoid arthritis. Cyclosporin A was purified in 1969 from a

culture of *Tolypocladium inflatum* obtained from a Norwegian soil sample. The fungus is a pathogen of beetle larvae. Though other fungi produce cyclosporin A, industrial production relies on productive strains of this single species. Cyclosporin A is an example of a non-ribosomal peptide that is synthesised by a huge multifunctional enzyme called **cyclosporin synthetase** (*simA*) that catalyses at least 39 different reaction steps. The enzyme is built from 11 protein modules and each of these functions in the recognition, activation, and modification of one intermediate. A small 12th module is thought to carry out the final cyclization step in cyclosporin A synthesis. The resulting structure of 11 amino acids is too complex to be produced by chemical synthesis, which means that improvements in production are reliant upon manipulation of the fungus. Methods that have been tested include changes in the carbon and nitrogen sources for the fungus, sequential addition of different carbon sources to its growth medium, addition of L-valine to the fermentation, immobilization of the fungus on beads, and cultivation on solid materials (solid state fermentation) rather than in liquid. Efforts to boost cyclosporin production have also involved the study of mutant strains of *Tolypocladium inflatum*.

Ergot Alkaloids

Ergot alkaloids, produced by the ergot fungus *Claviceps purpurea*, have poisoned human populations that consumed bread baked from contaminated rye flour and caused an incalculable number of deaths (Chapter 9). Used in a deliberate fashion as medicines, the same compounds have alleviated the suffering caused by migraine headaches, treated symptoms of Parkinson's disease, and stimulated uterine contractions and stemmed bleeding during childbirth. The toxicity and the therapeutic value of these compounds are due to their affinity for neurotransmitter receptors. The vasoconstriction leading to burning sensations associated with classic ergot poisoning, called St. Anthony's fire in the Middle Ages, is caused by the neuronal stimulation of smooth muscle contraction. The effectiveness of the ergot alkaloids in treating migraine and reducing bleeding lies in the same pharmacological mechanism. Other species of *Claviceps* produce ergot alkaloids and the same pathway for toxin synthesis is found in different members of the family Clavicipitaceae, including endophytes in the genus *Epichloë* (= *Neotyphodium*) that cause toxicosis in animals browsing on contaminated grasses (Chapter 9). The human pathogen, *Aspergillus fumigatus*, and related saprotrophs are also sources of these compounds. The relationship, if any, between the synthesis of the alkaloids and the development of various forms of aspergillosis is not known.

Ergot alkaloids are a diverse category of secondary metabolites that have been classified into three groups as clavines, amides of lysergic acid, and ergopeptines. Lysergic acid diethylamide or LSD is a synthetic derivative of lysergic acid, which is a key intermediate in the biosynthesis of ergot alkaloids. Synthesis of most of the ergot alkaloids involves a common set of initial reactions beginning with the addition of a pyrophosphate group to tryptophan followed by a series of steps to produce a tetracyclic ergoline ring. Genes encoding the enzymes for these pathways are organized in clusters. Ergotamine and ergometrine, produced by *Claviceps purpurea*, are the most important of the alkaloids used in medicine and a number of derivatives of these compounds have been adopted for treating specific conditions. Commercial fermentation is the main source of ergot alkaloids today.

Biotransformations

In addition to the value of natural and genetically modified strains of fungi as sources of pharmaceutical agents, fungi are used to modify the chemical structure of natural products including enzymes and small molecules produced by other organisms. These enzyme-catalysed steps include oxidation and reduction reactions, transfer of functional groups, and hydrolysis. Fungal transformations are used to produce anti-inflammatory cortisones from plant sterols, the hormonal contraceptive gestodene from other sterols, and ketoprofen, which is an important non-steroidal anti-inflammatory drug. Research on the synthesis of lethal amatoxins in species of *Amanita*, *Galerina*, and other mushrooms shows promise for future drug designs. The activity of these cyclic peptides is unaffected by cooking, stomach acidity, and digestive enzymes. Structural resilience, swift absorption, and precise targeting are among the preferred properties of new generations of chemotherapeutic agents for treating cancer and other illnesses. The design of these drugs may be aided by understanding the mode of action of amatoxins and the process of cyclic peptide synthesis in these mushrooms.

Biofuels

Fungi show tremendous potential for the production of biofuels from a variety of crop plants, including the vast quantities of rice straw and other kinds of **lignocellulosic agricultural waste**. This is a subject of considerable interest given our seemingly limitless need for combustible fuels, limitations to the availability of fossil fuels, the environmental damage caused by oil and gas extraction, and climatic consequences of burning these hydrocarbons. Once sugars are hydrolysed from the parent materials, or feedstock, their fermentation to alcohol by yeasts is a straightforward process. This is the source of bioethanol generated from sugarcane in Brazil and from corn in the United States. Brazilian sugarcane is milled to separate fibre residue from a juice containing 10–15% sucrose. The juice is filtered and concentrated to yield crystallised sugar and molasses; the sugar is refined for the food industry and yeast is used to ferment the molasses to produce ethanol. The fibre residue, called bagasse, is burned to provide the heat and electrical energy to power the carbon neutral – or, at least, energy efficient – biofuel plant. Corn ethanol production is more complicated from a biological point of view, because the corn seeds are rich in starch that must be hydrolysed into sugars prior to fermentation by yeast. This is achieved using purified glucoamylases and alpha-amylases. Some of these enzymes are derived from fungi, including *Aspergillus niger*.

The more significant industrial challenge comes from the treatment of agricultural waste to generate fermentable sugars from the complex polymers that form the bulk of these materials. This is referred to as **second generation biofuel production**, and the raw materials are called cellulosics and lignocellulosics. Rice straw contains a mixture of cellulose, hemicellulose, and lignin. Pre-treatment of the feedstock is designed to optimise the release of sugars and a variety of approaches have been tested. Grinding and milling of the rice straw increases the surface area for subsequent chemical reactions and mixing the straw with alkali and acids increases its subsequent digestibility. The effectiveness of white rot fungi, including *Phanerochaete chrysosporium*, *Pleurotus ostreatus*, and *Trametes versicolor*, in breaking down the various polymers has been tested. Rice straw treated with *Pleurotus ostreatus* for 60 days showed a 40% reduction in lignin content as well as the partial digestion of cellulose

and hemicellulose. Pretreatment of rice straw with *Aspergillus* and other fungi produced the highest ethanol yields, but none of the biological methods compared favourably with the effectiveness of chemical modification of the feedstuff. The discovery of new enzymes effective in lignocellulose degradation through genomic research is one area of research that may aid second generation biofuel production.

Bioremediation

Some of the basidiomycetes considered for second generation biofuel production have been selected as agents for the bioremediation of soil contaminated with **persistent organic pollutants** (POP). The reason that there is so much interest in this application of the white rot fungi is that the peroxidases and laccases that they secrete for lignin decomposition are also effective at degrading resistant aromatic contaminants that threaten human health. The target compounds include polycyclic aromatic hydrocarbons from the oil and gas industries; chlorinated compounds from wood preservatives, as well as discarded transformers and capacitors; halogenated compounds used as flame retardants, and nitroaromatic explosives used for mining and as weapons (Figure 12.10). Pesticides and other agrochemicals are another troubling source of soil contamination. White rot fungi are capable of breaking down compounds in all of these categories and impressive results have been obtained by inoculating sawdust and woodchips impregnated with petroleum hydrocarbons, TNT, chlorophenols, and polychlorinated biphenyls (PCBs). The efficacy of fungi in degrading these compounds in a natural setting is a subject of considerable commercial interest, but few large-scale studies have been published. Some of the largest trials have involved tubes of plastic mesh filled with pine bark and inoculated with fungi. These 'fungal tubes' are buried in the contaminated soil and aerated to promote growth. In addition to adding non-resident wood-decay and litter-decomposing fungi to contaminated sites, it may be possible to stimulate the activity of resident fungi by amending the soil with assorted nutrients. The addition of surfactants can



FIGURE 12.10 Oyster mushrooms (*Pleurotus ostreatus*) grown on soil contaminated with oil. The objective of this experimental form of bioremediation is to use the mycelium of the fungus to break down a variety of hydrocarbons in the oil and export the waste materials to the fruit bodies. If successful, harvesting and destruction of the fruit bodies would leave a cleaner soil. Source: <http://www.fungi.com/blog/items/the-petroleum-problem.html>.

increase the solubility of the contaminants and improve microbial access to the target chemicals. Another promising approach is to plant trees on contaminated sites and allow them to support mycorrhizal partners that are effective at cleansing the soil. The trees will recruit fungi from the existing soil microbiome and can be inoculated with specific mycorrhizal fungi before planting on the contaminated site.

Mycopesticides, Mycoherbicides, and Mycofungicides

Live fungi are used to combat insect pests of crop plants and to control the growth of weeds. *Beauveria bassiana* is an entomopathogen that infects a huge variety of insects and is used to control crop infestations by aphids, thrips, and whitefly. The fungus is cultured in solid state fermentation and formulations of its conidia are sprayed on plants as an emulsion or a wettable powder. *Metarhizium anisopliae* is a related ascomycete used as a mycopesticide against a variety of pests. One of the limitations of mycopesticides is that they do not work on contact, but require a few days to infect and kill the insect. Experiments to increase the virulence of *Metarhizium* include its transformation with a scorpion gene encoding a neurotoxin. A nice feature of this genetically modified fungus is that the neurotoxin gene is controlled by a promoter that is activated within the insect. This limits problems associated with human exposure to the transgenic fungus. Experiments on tobacco hornworm (*Manduca sexta*), and the mosquito, *Aedes aegypti*, which carries dengue and yellow fever, showed that the transgenic strain of the fungus was more effective at killing insects than the unmodified pathogen. *Beauveria* and *Metarhizium* belong to the Clavicipitaceae. *Lecanicillium lecanii* is a third fungus marketed as a biopesticide. It is a member of the family Cordycipitaceae.

Herbicides containing live fungi are called mycoherbicides. The fungi in these products are natural pathogens of the target weed plants. Fungi approved for use in mycoherbicides include *Alternaria destruens* for controlling dodder (*Cuscuta* species), *Colletotrichum gloeosporioides* for controlling low mallow and Virginia jointvetch (*Malva pusilla* and *Aeschynomene virginica*), and rusts (*Puccinia* species) that attack nut grass and woad (*Cyperus esculentus* and *Isatis tinctoria*). Chemical herbicides are very effective against weeds, but can persist in the environment and pollute groundwater. The development of herbicide resistance is another challenge associated with the use of conventional herbicides. Mycoherbicides are favoured by organic farmers who do not use synthetic herbicides. Potential uses of fungi for destroying opium poppy (*Papaver somniferum*), coca (*Erythroxylum coca* and *Erythroxylum novogranatense*), and marijuana (*Cannabis sativa*) have been investigated, but there is no evidence that drug control agencies have employed mycoherbicides. *Pleospora papaveracea* has been proposed as a mycoherbicide against Afghanistan's opium poppy crop. This fungus is a natural pathogen of poppies in Central Asia, which means that opium crops are already vulnerable to epidemic infection. This limits the value of *Pleospora papaveracea* as a mycoherbicide. Prospects for incorporating species of fungi that produce mycotoxins in herbicides are limited by concerns about the toxicity of these compounds toward humans and other animals.

Mycofungicides are a third category of agricultural product that contains live fungi. *Ampelomyces quisqualis* is a hyperparasite (parasite of a parasite) of powdery mildews whose potential as a biocontrol agent has been investigated for many years. A product containing spores of this species has been marketed as a biofungicide for treating powdery mildew of grape, strawberry, and roses.

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