THE MICROBIOLOGY OF BREWING

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HISTORICAL

Making beer is an ancient craft, but fermentation has been understood for only some 130 years. Yeast was previously regarded as undesirable scum and, with a few enlightened exceptions, brewers initiated fermentation fortuitously from yeast either clinging to badly-cleaned equipment or present in unsterilized wort. About 1836, sugar fermentation was ascribed to vital activity of yeast (33) and the fungal nature of yeast was recognized (201). At this time bottom-fermentation yeast was used only in Bavaria, but its use spread rapidly in Europe and then to the United States, because of emigra-

tion of many German brewmasters. Pasteur began his microbiological research, developed a reasonable theory of fermentation (182) and, in 1876, reported on beer spoilage by bacteria (183). Pasteur's work was extended by Hansen who developed methods for isolating single yeast cells and, from a selected cell, propagating a clone sufficient for commercial-scale fermentation (89). Modern developments in brewing microbiology relate to maintaining yeast free of bacteria and to rapid methods of fermentation.

ORGANIZATION OF THE BREWING INDUSTRY

The industry is well organized into national and international technical associations. Of particular note are 1. the American Society of Brewing Chemists, 2. the European Brewery Convention, 3. the Institute of Brewing (Great Britain and Australia), and 4. the Master Brewers' Association of America. Except for 3 international congresses are organized regularly. Collaborative research and the development of analytical methods feature in the work of 1, 2, and 3. At the present time, these organizations hope to establish universally accepted methods of analysis. Such developments are undoubtedly encouraged by the international sharing of technical knowledge, by companies building overseas breweries, and by amalgamation of brewery companies. The size of the industry can be judged by the estimated world production figure for beer or circa 5×10^{10} liters (231).

LITERATURE OF BREWING

There are many specialized journals devoted to brewing science and technology including: *Brauwelt, Brauwissenschaft, Bulletin of Brewing Science* (Tokyo), *Communications of the Master Brewers' Association of America, Journal of the Institute of Brewing, Proceedings of American Society of Brewing Chemists, Proceedings of the European Brewery Convention, Wallerstein Laboratory Communications.* Additional microbiological information relating to breweries appears in several international journals devoted solely to microbiology.

TEACHING AND RESEARCH IN THE INDUSTRY

Particularly in Europe, the brewing industry in individual countries has helped to organize centers of teaching and research in brewing science, usually in universities (130). In addition, Brewing Research Institutes have been set up in certain countries by national brewery associations and supplement the work carried out by the laboratories in individual companies.

BACTERIA ENCOUNTERED IN BREWERIES

The number of bacterial genera encountered in breweries is small. Gram-positive genera comprise *Lactobacillus* (frequent in top-fermentation breweries) and *Pediococcus* (more common in bottom-fermentation breweries). The gram-negative genera are virtually confined to *Aerobacter (Klebsiella), Acetobacter, Acetomonas, Obesumbacterium* and *Zymomonas.* Table 1 shows the stage of production at which they occur (3).

TABLE 1. BREWERY SPOILAGE ORGANISMS AND THE STAGE OF PRODUCTION AT WHICH THEY OCCUR (3)

Stage	Bacteria encountered
I Mashing and sweet wort	Thermophilic lactic acid bacteria (rare)
II Cooling of wort to pitching with yeast	Acetic acid bacteria (rare) Lactic acid bacteria (rare) *Obesumbacterium* (rare)
III Fermentation	*Obesumbacterium* Acetic acid bacteria Lactic acid bacteria
IV After-fermentation stages	Acetic acid bacteria Lactic acid bacteria *Zymomonas* (rare)

Ault, R. G. 1965. *J. Inst. Brew.* 71:376–91.

LACTIC ACID BACTERIA

Many species of *Lactobacillus* and *Pediococcus* have been claimed to be associated with brewing (12, 59, 63, 236) but it is possible that all of them may well be varieties of (*a*) *L. brevis* and *L. pastorianus* which are heterofermentative species with long rod-shaped cells, and (*b*) *P. damnosus,* a homofermentative species with coccal-shaped cells (42, 81, 230). Carbohydrate is degraded by the heterofermentative species by the phosphoketolase pathway (189). In contrast to homofermenters, the glycolytic pathway is inactive because aldolase and hexose isomerase are missing (250). The end products of metabolism include lactic acid, ethanol, glycerol, acetic acid, and carbon dioxide. Homofermenters produce lactic acid but some strains yield a small amount of diacetyl (9) which, at levels as low as 0.2 ppm, can spoil beer (a condition called sarcina sickness). A wide variety of carbon sources will serve, especially maltose (249) but some strains require in addition carbon dioxide (195).

Both homofermentative and heterofermentative strains need a wide range of amino acids, nitrogenous bases, and vitamins (162), and therefore growth in beer depends on incomplete uptake of these materials by the yeast. Growth of isolated strains is best in the pH range 5.0–6.5 but in the breweries the same strains may grow at pH values below 4.5. Some strains are introduced into the mash in Continental breweries in order to produce lactic acid and lower the pH of the wort: thermophilic strains such as *Lactobacillus delbrückii* are most suitable (229). Certain strains produce an extracellular or thixotrophic slime which is a heteropolymer containing glucose, mannose, nucleic acid, and sometimes protein (53, 246). This slime, or rope,

as it is called, spoils beer. Other undesirable effects of the bacteria upon beer include turbidity, acidity, and off-flavors (229). The bacteria may reinfect by persisting in the pitching yeast or in nonsterile equipment. Certain strains are capable of flocculating yeast (160).

Coliform Bacteria

These facultative anaerobes comprise strains of *Escherichia coli* and *Aerobacter (Klebsiella) aerogenes* that grow in wort rapidly, but in beer only when the pH is above 4.3 (229, 232). They produce a wide range of products of metabolism which impart flavors and odors to the wort that may be sweet and fruity, or resemble the smell of cooked cabbage. Coliforms may be introduced into the wort from water used for cooling or washing; they may be transmitted to the next fermentation via the pitching yeast but this is probably unusual. When wort is stored for continuous fermentation, coliforms may cause serious spoilage (3).

Acetic Acid Bacteria

When motile, *Acetobacter* has peritrichous flagella in contrast to the polar flagella of motile strains of *Acetomonas* (135, 207). The former genus has stronger powers of oxidation, the latter being scarcely able to oxidize ethanol further than acetic acid (190). *Acetobacter* strains may metabolize glucose via the hexose monophosphate pathway and the tricarboxylic acid cycle, but in some glucose is oxidized rather than phosphorylated (48, 242). In *Acetomonas* strains, the HMP pathway is normally used but the TCA cycle fails to operate due to lack of activity of isocitrate dehydrogenase and possibly other enzymes (245). There is therefore a requirement for certain amino acids or the corresponding oxo-acids. In contrast, *Acetobacter* strains can synthesize their entire complement of nitrogenous compounds from ammonia and suitable carbon fragments (25, 40). The simple nutritional requirements help to make them almost ubiquitous in some breweries and so become the most frequent cause of acidity, off-flavors, and turbidity (3). Certain strains are capable of causing yeast cells to die (71, 118). Acetic acid bacteria may also produce a dextranous "rope" in substantial quantities in beer (87). Frequently, the acetic acid bacteria grow as a greasy pellicle in order to increase exposure to atmospheric oxygen. Growth is very restricted in the absence of oxygen.

The variability of species of acetic acid bacteria is so marked that classification at this level may be of little value (206). A continuous spectrum of strains may exist with neighboring strains differing only in their ability to produce one or two enzymes.

Obesumbacterium proteus

This nonmotile species with short fat rod-like cells has the unique ability of growing in competition with actively multiplying yeast cells (209). It is a facultative anaerobe with a pH optimum of about 6.0 which grows with dif-

ficulty at pH levels below 4.5 (208). The glycolytic pathway, the HMP pathway and TCA cycle are active but terminal oxidation via the cytochromes is weak (221). Some strains at least require a spectrum of nitrogenous bases and amino acids for growth (214). It is not necessary for vitamins to be present in the growth medium and a large variety of sources of carbon will serve (214). The growth of the organism during a brewery fermentation depends on the strain of yeast used and, conversely, the rate of fermentation by the yeast is influenced by this bacterium (215). A characteristic odor of parsnips (204), possibly dimethyl sulfide (221), is transmitted to the beer by the metabolism of the bacterium. The following factors are important in the development of the organism in the early stages of fermentation: choice of yeast strain, seeding rate of bacteria, the pH of the wort, and the rate at which it falls during fermentation (35). If the numbers of *O. proteus* within the pitching yeast are high, the final pH of the beer will be greater than normal and the bacteria will be harvested in still larger numbers in the yeast crop (35).

Zymomonas anaerobia

The strains are usually highly motile with rod-like cells bearing 1–5 polar flagella (205). The species is a strict anaerobe and can grow over a wide pH range (3.5–7.5). Glucose and fructose, sometimes sucrose present in beer, but not maltose, are utilized as carbon sources (11). Energy is provided by the Entner-Doudoroff pathway, and the end products are ethanol and carbon dioxide (155). Minor products such as hydrogen sulfide and acetaldehyde give a highly objectionable odor (205). The organism grows in beer sweetened with sucrose or invert sugar and is transmitted by nonsterile equipment (3).

WILD YEASTS

Wild yeasts are those strains that are present in wort, beer, or other brewery materials which, by their action, do not enhance, and often spoil, the final products. Many wild yeasts are strains of *Saccharomyces cerevisiae* or *S. carlsbergensis* and cause off-flavors, fermentation of dextrins, and turbidity of beers (244). They are difficult to distinguish from culture yeasts on morphological and physiological grounds but very recent work with selective media has been encouraging (85). Serological methods have been used routinely to identify them in pitching yeasts (193). The current methods are based on antigenic relationships within the genus *Saccharomyces* (34, 199), and in the case of top yeasts, the antiserum to *S. pastorianus* is obtained from rabbits. This is absorbed with the culture yeast used in the brewery and then used for treating samples of pitching yeast under test. The wild yeasts may be present in proportions as low as a few cells per million cells of culture yeast (192), but the antiserum may be located around the wild yeasts by using an antirabbit serum from goats which has been coupled to a fluorescent dye such as fluorescein. A suitable microscope

with ultraviolet illumination permits an operator to distinguish the fluorescing cells of the wild yeast (194).

Surveys of wild yeasts have been carried out in Britain on raw materials (244), pitching yeasts (18), and on draught beers (98). Apart from wild yeasts of the genus *Saccharomyces,* representatives from other genera such as *Candida, Debaryomyces, Hanseniaspora, Hansenula, Kloeckera, Pichia, Rhodotorula,* and *Torulopsis* have been encountered. Species of *Saccharomyces* fail to grow on a medium in which lysine is the sole source of nitrogen, while those of other yeast genera usually do, and therefore a lysine test enables the brewer to identify certain wild yeasts in low numbers in the presence of large numbers of culture yeasts (163).

OUTLINE OF TRADITIONAL BREWING PROCESSES

Traditional brewing processes include (*a*) malting, a process whereby barley is germinated, forming malt, thereby increasing the array of enzymes necessary for converting various substances in malt and malt adjuncts to forms capable of assimilation by fermenting yeast; (*b*) brewing of wort includes cooking, mashing, wort filtration, kettle boiling, and wort cooling; (*c*) fermentation of wort; (*d*) aging of fermented beer; (*e*) finishing and filtration; and (*f*) beer packaging.

Due primarily to space, equipment, and technical know-how, brewers, except for some of the largest ones, depend on the maltster for finished malt. As a prelude to successful malting, a pure variety of barley must be selected to ensure evenness of germination during malting (46). The major malting steps include grain storage to insure proper dormancy, steeping of the grain to acquire the necessary moisture content for malting, malting of the grain to prepare the malt, and kilning of the malt which serves to arrest germination and modification and provides malt with its characteristic flavor. Kilning of the malt is divided into two distinct phases: (*a*) drying which is the last step in germination, and (*b*) curing which is essentially a physicochemical reaction between different malt constituents. The manner of controlling these two phases helps determine whether the final malt will be a pale or dark malt (46). In all of the above malting steps, careful regulation of temperature, moisture, and aeration are important to produce properly modified malt. Related microbiological problems are discussed under the microbiology of brewing materials.

The malt as delivered to the brewery is next ground for efficient release and extraction of nutrients therein during mashing. Most American beers, in addition to malt, contain a starchy adjunct, usually corn or rice, which must by hydrolyzed to simpler sugars via amylolytic enzymes present in malt. In contrast, adjuncts are not permitted in German beer. The adjunct is first gelatinized by boiling in a cooker with a small amount of ground malt. Following gelatinization, the cooker contents are transferred to a mash tun which contains the bulk of the ground malt mixed with water. The primary objective of mashing is enzymatic conversion of the majority of starches,

proteins, lipids, and organic phosphates into simple water-soluble forms. The resultant extract is named wort. The mashing temperature(s) and pH are dependent on the particular mashing process. The major mashing processes are infusion, decoction, and mixed mashing which is a combination of the first two. Infusion mashing is used for ale, whereas decoction mashing is used for lager beer and mixed mashing for production of Lambic and other old beer types (46). A primary difference between infusion and decoction mashing is boiling—in infusion mashing the temperature is held constant without boiling, whereas in decoction mashing a part of the mash is withdrawn, boiled and returned to the mash tun to raise the temperature of the whole mash. Boiling is not necessary for infusion mashing since highly modified malts are used which are already partly peptonized. Furthermore, a low proportion of unmalted cereal adjuncts are used and these are often precooked flakes.

Following mashing, the wort is separated from the spent grains and boiled in a kettle. Wort separation is essentially a filtration process which can be accomplished by two different methods. For ale production the mash tun is used for both mashing and filtration, whereas in lager beer production, filtration is almost always carried out in a separate vessel, either a lauter tub or a mash filter.

Major reasons for wort boiling are wort sterilization, enzyme destruction, coagulation of unstable colloidal protein named trub, and extraction of bittering substances and essential oils from hops. Boiled wort does contain viable *Bacillus* and *Clostridium* spores which, due to the acid environment, cannot propagate therein (3). In addition, hop resins contain substances toxic to many Gram-positive rod-shaped bacteria. According to Wackerbauer & Emeis (239), all types of lactobacilli can grow in unhopped beer but only a few can grow in the presence of hop resins. Unfortunately, the hopping rate in many countries is too low (e.g. ca 0.3 lb per bbl in the United States) to be of bactericidal value.

After boiling, spent hops are extracted by passage of the wort through a strainer known as the hop jack. The wort is next pumped to a closed hot wort tank followed by centrifugation to remove one form of proteinaceous particle called "hot trub" which coagulates in hot wort. In Britain, whirlpool-type hot wort tanks are often used for removing hot trub, thereby obviating the need for centrifuges. Wort also contains protein particles called "cold trub" which coagulate only in cold wort. The latter particles are probably structurally related to another proteinaceous material called "chill haze" which often forms in previously chilled warm beer. In former years an open tank, a coolship, was used for wort cooling, thereby enabling precipitation of cold, as well as hot trub particles. The wort is next cooled to pitching temperature by passage through a cooler, where air is simultaneously injected to help reduce the lag phase of yeast growth. Some breweries even use a limited additional aeration upon initiation of fermentation. One danger in the latter procedure is that of inducing excess diacetyl pro-

duction (186). In a modern brewing operation all of the above processes from kettle boil to fermenter delivery occur in a closed system thereby helping to assure wort sterility at the onset of fermentation.

The two major beer types are lager and ale which are fermented with bottom and top yeasts, respectively. Most bottom yeasts are *Saccharomyces carlsbergensis* species whereas most top yeasts are *S. cerevisiae* species. Bottom yeasts flocculate and settle out to the fermenter bottom near the end of fermentation, whereas top yeasts remain powdery and form a yeasty head on the wort surface. As a consequence thereof, top yeasts are collected for repitching from the surface layers of the beer, whereas bottom yeasts are collected from the fermenter bottom. Lager beers are produced throughout the world, whereas ale is restricted primarily to countries of British origin. Some American breweries producing both lager and ale use only one yeast, a bottom yeast, for both beer types, thereby eliminating the problems involved in propagating and keeping two yeast cultures separate (188). Such ales produced with bottom yeast are designated "bastard ales." Conversely, in England, a "bastard lager" is produced from infusion wort plus bottom yeast. A lager yeast used to ferment ale is usually not repitched because of enhanced autolysis due primarily to the higher fermentation temperatures (15–20°C) of ale as compared to lager beer (6–11°C). An additional consequence of the higher fermentation temperature is a shorter fermentation interval for ale (4–5 days) as compared to lager beer (6–7 days). Melibiose fermentation is a diagnostic test used to separate the two yeasts. *S. carlsbergensis* is melibiose-positive, whereas *S. cerevisiae* is usually negative (191). In addition, *S. cerevisiae* usually sporulates more readily then *S. carlsbergensis*.

Following primary fermentation, the beer is subject to various aging (lagering) and filtration procedures depending upon the beer type and available facilities. In most instances, the beer is cooled to approximately 0°C near the end of primary fermentation and kept at that temperature until packaged. The low temperature helps retain carbonation and prevent microbiological contamination. Some major objectives in transferring beer to aging tanks following primary fermentation are: (*a*) beer clarification will hopefully occur through precipitation of most of the remaining yeast and cold trub. (*b*) The low storage temperature also helps precipitate a proteinaceous chill haze complex so as to obtain a more brilliant beer, as well as prevent subsequent haze formation when the beer is chilled after bottling. (*c*) Aging helps improve beer flavor by various, as yet little understood, chemical changes. (*d*) Some brewers use the aging period as a means of saturating the beer with carbon dioxide through secondary fermentation. Other brewers subject the aged beer to a primary filtration and then saturate the beer artifically with carbon dioxide in finishing tanks. Purging with CO_2 also helps wash undesirable volatiles out of the beer.

After finishing, the beer is subject to a secondary filtration followed by

transfer to bottling tanks. After filling, package beers are often pasteurized in conventional tunnel pasteurizers.

OUTLINE OF ADVANCED BREWING PROCESSES

Dry milling of malt requires complex precision equipment to achieve uniform trituration of the endosperm to coarse flour and, at the same time, preserve the husks almost intact (46). In several breweries, the husk is rendered less brittle by either brief steaming or steeping. Usually steeping is followed by wet milling to produce a mash and therefore a separate mash-mixer is not required (132). In order to reduce the requirement for mashing equipment capacity, syrups resembling concentrated sweet wort are used by breweries in several countries (149). The syrups may be derived from many unmalted cereals, but most commonly from maize and barley, and the degradation is achieved by industrial amylolytic enzymes (142). In the case of maize, acid hydrolysis may precede enzymatic degradation (46). Hops are bulky and are usually stored in cold-rooms; they are replaced in many breweries by a vacuum-packed powder produced by milling the hops (14) and selecting a fraction rich in lupulin glands, the site of bitter resins and essential oils (30). Alternatively, the resins may be extracted by an organic solvent to yield a semisolid that may be canned (159). In the copper (kettle) the principal bitter resins are isomerized to isohumulones (212). Similarly, the extract may be isomerized by boiling with dilute alkali (107). This preparation is usually added after fermentation in order to obviate adsorption of the isohumulones to precipitated protein in the copper and yeast in the fermenter (50).

Enclosed fermentation vessels are replacing open ones because the former are more easily spray-cleaned, have reduced susceptibility to air-borne microbial infection, are cooled more readily and facilitate collection of evolved carbon dioxide (233). In cylindro-conical vessels, which are a popular type of enclosed fermenters, the yeast separates from the beer at the end of fermentation into the cone from which yeast slurry can easily be removed (238). Under slight pressure, the carbon dioxide produced from fermentation will readily dissolve in the beer. Large open-air vessels, now used in some breweries for fermentation and beer storage, may be built and maintained cheaply for their capacity (220). The speed at which yeast is naturally separated from beer has, in the past, determined to some degree the duration of the beer in fermenters, but centrifuges are now commonly used to achieve the separation (37).

Continuous fermenters recently introduced have included stirred vessels coupled in series which are superior in performance to a single stirred vessel (15, 105). Yeast recycled, from the emerging beer, to the fermenters increases the overall fermentation rate but at high yeast concentrations the yeast cells divide slowly and the quality of the beer produced is altered (104). Unstirred continuous fermenters of tower form permit dwell-times

in the order of 6–9 hr and are used commercially (4, 198). Semicontinuous or accelerated batch systems have been used successfully on at least pilot scale (83).

MICROBIOLOGY OF BREWING MATERIALS

The prime ingredients in beer are water, malt, hops, and yeast. Some European beers are all malt beers, whereas most American beers contain ca 50 per cent malt and 50 per cent adjunct, usually corn or rice.

Production of suitable brewing water is usually not a major problem since it can be treated to either add or remove various metal ions and salts (39, 46). It is an almost universal practice to harden or mineralize the mash water or mash with calcium and magnesium sulfates, thereby lowering the pH of the mash. A high pH is unfavorable for a number of important reactions in the brewing process, e.g., at mashing saccharification will not proceed smoothly, the coagulation of protein (trub) at boiling is incomplete, and the resultant beers with a high pH are biologically unstable and liable to infection with lactic acid bacteria (46). Furthermore, the damaging effect of nitrates on fermentation is diminished (235). Other effects of inorganic ions, e.g. zinc, are discussed in the section on growth and fermentation kinetics.

Also of importance is the requirement that the water supply be relatively free of decaying vegetation, e.g., algae, and industrial wastes such as phenolics (241), since these can contribute off-tastes and odors that are difficult to remove (157).

Production of suitable malt begins on the farm where a pure variety of barley is necessary to obtain evenness of germination during malting (46). In moist growing seasons the barley may become infected with *Fusarium* spores that grow during the steeping process, producing substances which cause gushing in beer (73). Growth of foetid bacteria on the husks during steeping also occurs and can be eliminated in part by frequent changing of the steep liquor (46). Mold growth during steeping is reduced by raising the pH of the steep liquor with lime (46). Unfortunately, this process encourages growth of coliform-type bacteria (203). Another microbiological problem relates to the use of gibberellic acid to speed up germination during malting. The malts produced thereby have higher soluble N content which encourages growth of thermophilic lactobacilli during mashing (189). In certain cases, this might be desirable, particularly in countries such as Germany where the use of acids is prohibited in brewing liquor (171). Recent studies (61, 252) emphasize the importance of having the proper amount of amino acids in wort for achieving a satisfactory fermentation. The composition thereof is dependent both on the quality of the raw materials and mashing procedure (61, 184).

Hops, in addition to antiseptic value (239), contribute to the foam-stabilizing properties of wort and are important for achieving satisfactory yeast head production in ale-type beers (51). Current interest in fermenting un-

hopped wort with the subsequent addition of a preisomerized hop extract may complicate this problem (50).

SELECTION AND PROPAGATION OF BREWERS' YEAST

It is usual to select strains of yeast for brewing from yeasts already in commercial use. While the application of genetic principles to the production of new strains of bakers' yeast has been successful (65), there have been few instances of induced hybridization for commercial brewing (112). Mutation and transformation (178) have also been suggested for producing brewing strains with new properties but there has been no commercial exploitation. Desirable features in a brewing yeast include (*a*) the capacity to produce a beer of good flavor and aroma; (*b*) the ability to ferment wort rapidly until fructose, glucose, sucrose, maltose, and maltotriose have been used; and (*c*) the propensity to grow in wort rapidly until, in normal batch fermentation, the total concentration of yeast is in the range 2.5–5.5g dry weight/liter. In batch fermentation, it is desirable that the yeast separates readily from the beer at the conclusion of fermentation although less necessary if centrifuges are used for separation (37). Selection is normally based on the results of small-scale fermentations (213). For continuous fermentation using unstirred towers, it is necessary to have a yeast which is strongly sedimentary throughout the fermentation in order to maintain a yeast plug at the base of the tower (4, 32, 198).

Some breweries isolate, select and maintain their yeast strains but others engage specialist laboratories to provide this service. The entire yeast within a brewery may be derived from a single cell, from several isolated cells, from a single yeast colony or from several colonies (32). Again, some breweries choose to have two or more strains that may be employed in mixture or in separate fermentation vessels. Proportions of strains in a mixture may, however, change because of alterations in materials or procedure, and individual strains may be eliminated (103). Nevertheless, a yeast of several strains may adapt more successfully than a single clone. Cultures may be maintained at 10°C on wort-agar slopes or at 4°C in carbohydrate media such as 10 per cent sucrose, wort, or Wickerham's malt extract medium (243). Subculturing is carried out at regular intervals (24), preferably at less than three-month intervals. Lyophilized cultures have not been used extensively because there is a high mortality of cells during freeze-drying, and thus mutants and variants may be selected (251).

Many yeast propagators are based on the pioneer work of Hansen and Kühle and operate either semicontinuously or on a batch basis (88). Sterile wort is run into a vessel that has been presterilized by steam and cooled. Sterile air or oxygen is perfused through the wort, and the culture of yeast from the laboratory is inoculated. Aeration or oxygenation may be supplied continuously but, because of foam formation, it is more usually intermittent (43). In a modern example, the propagator is charged with 23 hl of wort of specific gravity 1.040 and is pitched with 91 g of pressed yeast;

aeration is provided for 1 min in every 5 at 0–11 m^3/ minute. At approximately 18°C exponential growth occurs for 48 hr when some 54 kg of pressed yeast is available in partly fermented beer of specific gravity 1.016. When the entire contents of the propagator are discharged into 250 hl of fresh wort there is no lag phase (5, 222). With some strains of yeast, the pH levels of beers produced in the propagator are low and the cells are elongated, but these effects are lost when the yeast is used normally (43). Modern cylindro-conical fermenters may be used as yeast propagators and stirred-tank continuous fermenters are particularly good (62).

Propagation of brewers' yeast enables a brewery to replace the entire stock of yeast on a predetermined basis. Frequently, a batch of yeast is used only about 12 times before it is discarded. There are, however, breweries claiming that their yeast has not been changed for 50 years or more (139). The changes in a yeast that persuade brewers to discard them relate either to infection with bacteria or wild yeast, poor settling near the end of fermentation if a bottom yeast, or partial loss of ability to grow, ferment, and produce the expected quality of beer.

YEAST MANAGEMENT

In the average brewery, a large inoculum of cells is used (ca 5–15 million cells/ml of wort). In each fermentation the number of cells increases three- to fourfold. Therefore, one-third to one-fourth of the yeast crop of each fermentation is used for inoculation of the next batch. If the brewery is of sufficient size (ca 1 million bbls of beer or more annually in the United States), drying of the remaining yeast for use as an animal food supplement becomes economically feasible. Alternatively, the yeast is used for manufacture of yeast extract or for fermentation in grain distilleries.

Yeast collected for repitching is usually mixed with 2–3 volumes of chilled water and passed through a vibrating screen to help remove bitter cold trub particles (196). In a modern brewing operation, the screened yeast passes directly into a scale hopper thereby providing the required amount of yeast for repitching (Editorial 1959, *Brewers Digest* 24:11). One danger in washing with water is a change in metabolic activity from fermentation to respiration (31), thereby increasing susceptibility to autolysis (116). Conversely, storage under chilled water is believed to hold autolysis to a minimum (100). Yeast to be stored for a prolonged period of time is best left in the fermenter under beer (38). One danger of prolonged storage is incomplete ability to ferment upon reuse (197). A minimum 24-hr rest period is believed necessary before reusing a yeast (197), but present practice in Britain with top and bottom yeasts in cylindro-conical vessels belies this belief.

Some suggestions for reducing yeast autolysis include iron enrichment and maintenance of a high C to N ratio (117), and the addition of unsaturated fatty acids to wort (223, 224). An important index of yeast autolysis is increased proteolytic activity (10).

Yeast contaminated with beer spoilage bacteria may either be replaced

with a pure culture or washed with acids such as phosphoric acid (45), ammonium persulfate (27), or a combination thereof (7), thereby eliminating the necessity for replacement. Yeast replacement or acid washing can affect beer flavor since it usually requires several fermentations for fresh yeast to become acclimatized to the brewery (16). Related information on yeast replacement and acid washing is found in sections Selection and Propagation of Brewers' Yeast and Microbiological Control in Brewing, Fermentation, and Packaging Including Sanitation.

GROWTH AND METABOLISM

Brewers' wort (145) commonly has 8–14 per cent total solids, of which 90–92 per cent are carbohydrates. The major carbohydrate components of wort are glucose, fructose, maltose, sucrose, maltotriose, and a group of linear and perhaps also branched polymers of glucose containing four or more units. Brewers' yeast uses the sugars up to maltotriose but not the larger molecules (91). More fermentable worts are produced if the malts used are rich in amylolytic enzymes; unkilned malts are particularly rich. Lowering the mashing temperatures increases fermentability (86). Raising the proportion of unmalted cereal or the temperature of mashing diminishes wort fermentability (13, 110). Similarly, the concentration of nitrogenous material in the wort is influenced by the malt and other materials used in wort-making and by mashing and wort boiling conditions (109, 200). Commercial worts commonly have 70–110 mg N/100 ml, and the nitrogenous constituents include ammonia, simple amines, amino acids, purines, and simple peptides to complex proteins (145). The most important source of nitrogen is the amino acids. Proline, an imino acid, is abundant but is scarcely used (113). Biotin, inositol, pantothenic acid, pyridoxine, and thiamine are present in wort and utilized by brewers' yeast. The total ash content of wort represents about 2 per cent of the wort solids; phosphates, chlorides, sulfates and other anions are present with the cations Na, K, Ca, Mg, Fe, Cu, and Zn. Phosphate content is in the range 60–120 mg/100 ml (64), and sulfate content in the region of 400 mg/liter (125). Dissolved oxygen content varies from about 4–14 mg/liter (154).

The growth and metabolism of brewers' yeast have recently been reviewed (191). Yeast cells readily take up monosaccharides by facilitated diffusion (120) but di- and trisaccharides enter the cell by means of a permease system (92, 93) which is inducible in some strains, constitutitive in others. Maltotriose is the last fermentable carbohydrate to be taken up. There is also a sequence of uptake of amino acids (Table 2) probably because of competition at the permease sites between the various acids (113, 114). The yeast is able to synthesize certain amino acids more easily than others. Thus, lysine, histidine, arginine, and leucine yield oxo-acids which are not furnished to any extent from carbohydrate metabolism and therefore changes in their concentration may affect the general metabolism of the yeast and hence the quality of the final beer. Nitrogen nutrition is complicated, however, by the ability of yeasts to release amino acids and nucleo-

TABLE 2. ORDER OF ABSORPTION OF AMINO ACIDS FROM WORT BY BREWERS' YEAST (113)

Group A	Group B	Group C	Group D
Immediately absorbed	Absorbed gradually during fermentation	Absorbed after a lag	Only slowly absorbed after 60 hr
Arginine	Histidine	α-Alanine	Proline
Asparagine	Isoleucine	Ammonia	
Aspartate	Leucine	Glycine	
Glutamate	Methionine	Phenylalanine	
Glutamine	Valine	Tryptophan	
Lysine		Tyrosine	
Serine			
Threonine			

From Jones, M., Pierce, J. S. 1964. *J. Inst. Brew.* 70:307–15.

tides into the medium especially when changing the medium, thereby causing alterations in membrane permeability (49, 136).

When yeast is pitched into aerated or oxygenated wort, there is at first a lag period when the cells actively take up materials from the wort, including the dissolved oxygen. It is not certain why the oxygen is important for the growth of the yeast but it may well permit synthesis of unsaturated lipids (2, 23) and influence mitochondrial function (36). The level of oxygen (about 4–14 mg/liter) is insufficient for any significant aerobic respiration and indeed the high levels of fermentable sugar ensure by the Crabtree effect (47, 211) that the metabolism is anaerobic. The major energy-yielding pathway is the glycolytic Embden-Meyerhoff-Parnas (EMP) one, but the hexose monophosphate shunt mechanism operates to a limited extent, mainly for the synthesis of pentoses (102). Pyruvic acid, the product of the EMP pathway, undergoes enzymic decarboxylation and reduction to ethanol and carbon dioxide. While this is the outstanding feature of yeast metabolism during beer production, special flavors and aromas of beers may arise from minor biochemical reactions (Table 3), notably those stemming from pyruvic acid. For instance, esters arise from an intracellular reaction involving acyl-CoA compounds, alcohols, and ATP (175). Ethyl acetate is thus produced from acetyl-CoA and ethanol, both products of pyruvic acid metabolism. The various fatty acids available within the cell compete in ester synthesis, except that propionic, isobutyric, and isovaleric acids do not furnish ethyl esters. Leakage of acyl CoA-compounds from the synthesis of higher fatty acids may also contribute to the level of esters, for instance, ethyl caprylate (174).

Esters other than ethyl esters utilize fusel alcohols which arise from either carbohydrate or amino acid metabolism, giving a range of oxo-acids (6, 106, 111, 115). Oxo-acids in excess of the requirements of the yeast may

TABLE 3. TASTE THRESHOLDS OF SOME BEER CONSTITUENTS (μG/ML)

	In water	In lager beer	In degassed beer
Methanol	36.9	—	100
Ethanol	8.20	—	—
Propanol	6.08	—	50
Isopropanol	6.01	—	100
2-Methylpropanol	0.565	—	100
2-Methylbutanol	4.15	—	50
3-Methylbutanol	0.291	—	50
β-phenylethanol	0.00317	47.9	50
Ethyl acetate	0.257	93.5	5
Butyl acetate	0.043	2.63	—
Isobutyl acetate	0.073	—	1
Amyl acetate	0.009	3.44	—
Isoamyl acetate	0.019	2.30	1
Diacetyl	0.00261	0.162	0.005

From the results given in References 96 and 202.

be enzymically decarboxylated to the corresponding aldehyde which is then reduced to yield the fusel alcohols (Table 4). Thus, the uptake of isoleucine, leucine, valine, and phenylalanine from wort results in production by the yeast of 2- and 3-methyl butanol, iso-butanol and phenethyl alcohol. The choice of yeast strain, conditions of fermentation, and wort composition each affect fusel alcohol formation, thereby modifying beer flavor and aroma and providing material for ester synthesis.

Acetoin, diacetyl, and 2,3-pentanedione are normal beer constituents but in excess they spoil the beer by their musty, buttery, and honey flavors, respectively. The threshold of tolerance for vicinal diketones is in the order of 0.2–0.5 μg/mg (52, 240). Acetoin is produced from "active acetaldehyde" (hydroxyethyl-2-thiamine pyrophosphate) and free acetaldehyde in the presence of a carboligase. Yeast does not oxidize acetoin to diacetyl but instead tends to reduce diacetyl; thus, yeast is often added to filtered beer if the level of vicinal diketones is too high. Active acetaldehyde will react with pyruvic and oxo-butyric acids to yield acetolactic and acetohydroxy butyric acids, respectively, and it is believed that these acids (which may be precursors of valine and isoleucine) diffuse to some extent from the yeast cells into the beer. By decarboxylation and oxidation within the beer, the vicinal diketones are produced (218). Strains of *Pediococcus* and respiratory-deficient mutants of brewers' yeasts are sometimes responsible for high levels of vicinal diketones (44).

Yeast requires sulfur for the production of proteins, coenzymes, vitamins, etc., and takes up organic sulfur from wort, chiefly as methionine, and inorganic sulfur in the form of sulfate (152). Hydrogen sulfide is generated during yeast metabolism and depends, in brewery fermentations, on the

TABLE 4. ALCOHOLS, ALDEHYDES, OXO ACIDS, AND AMINO ACIDS IDENTIFIED IN YEAST (217)

Alcohols	Aldehydes	Oxo acids	Amino acids
Ethanol	Acetaldehyde	Pyruvic acid	Alanine +
Glycol	Glyoxal	Hydroxypyruvic acid	Serine
Propanol	Propionaldehyde	α-Oxobutyric acid	α-Aminobutyric acid
Isopropanol	—	—	—
Butanol	Butyraldehyde	—	—
Isobutanol	Isobutyraldehyde	α-Oxoisovaleric acid	Valine
Sec. butanol	—	—	—
Tert. butanol	—	—	—
Isoamyl alcohol	Isovaleraldehyde	α-Oxoisocaproic acid	Leucine
Act. amyl alcohol	Act. valeraldehyde	α-Oxo-β-methyl valeric acid	Isoleucine
Hexanol	Hexanal	—	—
Heptanol	Heptanal	—	—
—	—	Oxalacetic acid	Aspartic acid
—	—	α-Oxoglutaric acid	Glutamic acid
Phenethyl alcohol	—	Phenylpyruvic acid	Phenylalanine
Tyrosol	—	Hydroxyphenyl pyruvic acid	Tyrosine
Tryptophol	—	—	Tryptophan

Suomalainen, H. 1968. *Aspects of Yeast Metabolism*, 17, ed. A. K. Mills, H. Krebs. Oxford: Blackwell.

yeast strain used, the temperature, and the wort composition (123). The gas, unpleasant over certain threshold levels, arises either from leakage of sulfide ions during the enzymic reduction of sulfate or more likely by the action of cysteine desulfhydrase on cysteine (133). Mercaptans, sulfides, and thicarboxyls have been implicated in the flavor of beer (227). Nevertheless, growth of yeast in synthetic media and wort gives rise to no significant levels of volatile organic sulfur compounds (97, 176). These compounds arise from nonenzymic reactions in the beer (170) and from the metabolism of spoilage bacteria (1).

GROWTH AND FERMENTATION KINETICS

Brewery fermentations are characterized by the use of a complex medium and a large inoculum. In batch fermentations, the pitching rate is in the order of 0.2–0.4 mg dry wt/ml and the final harvest is 5–10 times this amount, depending on the yeast strain used, the composition of the wort and the process conditions that apply. Variations in pitching rate strongly influence the time to achieve fermentations (237) and in the ability of the yeast to utilize maltose (79). The small number of cell divisions normally occurring usually means that a true exponential growth phase is absent and is

replaced by an almost linear increase in cell mass. Arrest of growth often occurs because there is an insufficiency of assimilable carbohydrate but may also occur if the yeast has come out of suspension due to premature sedimentation (bottom yeast) or premature yeast-head formation (top yeast). Rates of utilization of maltose and maltotriose by individual yeast strains appear to be dependent on the malto-permease system, and not on the overall maltase activity (79). Levels of pH and various cations, including K^+, Zn^{2+}, Mg^{2+}, and NH_4^+ appear to be important in utilization of maltose and maltotriose (234). The importance of Zn^{2+} levels in wort in influencing the quantity of yeast crop is recognized (147).

The role of oxygen has been mentioned earlier in connection with the Crabtree effect. With growth and fermentation kinetics, the continued use of wort containing 0.5 ppm dissolved oxygen immediately before pitching (6 per cent of air saturation level) leads to progressively poorer fermentations (108). In the range 0–20 per cent saturation of wort by oxygen before pitching, dissolved oxygen concentration is directly proportional to yeast crop and to fermentation rate (150).

YEAST CELL WALL

The brewing yeast cell wall is fairly rigid, layered, 100–200 nm thick, comprising glucans (40 per cent), mannans (40 per cent), protein (8 per cent), lipid (7 per cent), inorganic material (3 per cent), hexosamine and polymers (2 per cent). These proportions are approximate and vary according to yeast strain, cell age, and growth conditions (80, 156). The wall has associated with it, in free and bound forms, invertase, acid phosphatase, catalase, proteases and, in the case of *S. carlsbergensis*, melibiase (129, 146). Glucamylase is present in the wall of *S. diastaticus*, a wild yeast (101) and hydrolyzes beer dextrins. Other extracellular enzymes in the wall include glucanases (148), mannanases (148), protein disulphide reductases (173) which, with the proteases, are probably responsible for hydrolyzing the wall (161) and thus influence the budding process and the final shape of the bud (22). In top fermenting yeasts, certain strains are characterized by buds failing to detach so that chains of cells are formed (26).

The cells of other brewing yeasts may clump together to form flocs, especially in the absence of sugars and in the presence of divalent cations (54). The mechanism of flocculation probably involves the creation of salt bridges between superficial phospho-mannan-proteins of adjacent cells (141), and hydrogen bonding stabilizes the bridging (95, 158). The creation of flocs is important in beer clarification by sedimentation or centrifugation. Most strains of brewing yeast have a strong negative charge because of superficial phosphate and carboxyl groupings (55) and will therefore react with positively charged fining agents such as collagens (134) and certain gelatins. The ability of top fermentation yeasts to form a yeast-head (51) is also a function of the cell wall surface but the substances responsible for holding the cells at a gas/liquid interface have not been identified.

BREWING YEAST GENETICS

Recent reviews by Matile, Moor & Robinow (151), Fowell (67), and Mortimer & Hawthorne (165) provide timely information on three interrelated subjects: yeast cytology, sporulation, and genetics, respectively. Brewing yeast genetics, although moving at a steady pace, has been retarded somewhat because of the following: (*a*) One of the most important but least understood variables, the effect of yeast on beer flavor, is not yet amenable to genetic control (247). Some factors which are, include flocculation (69, 225), fermentation rate and limit (72, 228), maltose (78, 185, 228), maltotriose (228), and dextrin fermentation (68, 228), and total yeast cell count and cell size (69, 123). (*b*) The brewing characteristics of laboratory yeast hybrids are not always the same when evaluated in different breweries, indicating thereby that the environment plays a role (60). Also desirable for brewing is the development of new strains for use in continuous brewing and fermentation (143). (*c*) Most industrial yeasts are polyploids which sporulate poorly (57, 248). Associated therewith is low ascospore viability (56). This approach for altering the genotype can be obviated somewhat by induction of mitotic haploidization (58). (*d*) Mutants produced artificially have not produced suitable brewing yeasts (228). This is not surprising since most mutations are deleterious. Other methods for altering the genotype which may prove more beneficial include mitotic crossing-over, gene conversion, and nondisjunction (67). These occur at a higher frequency than mutation and can sometimes be induced by chemicals such as *p*-fluorophenylalanine. Another promising approach is transformation (178, 179), although to date it has not proven to be successful with yeast (94, 131, 164).

MICROBIOLOGICAL CONTROL IN BREWING, FERMENTATION, AND PACKAGING INCLUDING SANITATION

In general, methods for microbiological control have not changed significantly during the past 30 years except where dictated by a change in beer processing. Major changes in beer processing include the use of closed as compared to open vessels for brewing, fermentation, aging and packaging, attendant therewith is the adoption of in-place cleaning systems, the production of nonpasteurized package beer, and continuous brewing and fermentation. The first two processing changes help reduce bacteriological contamination, thereby lessening the need to examine more and more samples. Two additional parameters worthy of consideration in choosing a microbiological quality control program are: (*a*) Use of standards whenever possible, e.g., in a fermenting beer what constitutes an acceptable level of contamination? This will vary depending upon the overall design and construction of the brewery. Thus, open fermenters in older breweries with wooden surfaces are difficult to clean and will no doubt have a considerably higher level of contamination than modern breweries with closed fermenters with easy-to-clean rounded surfaces such as stainless steel. (*b*) Common sense realism is

necessary, e.g., a beer to be pasteurized or a draft beer to be consumed within a short period of time may contain a small number of organisms, whereas a beer subject to aseptic filling can withstand very few, if any, contaminant organisms.

Some of the more recent survey papers on microbiological control are listed herein (17, 41, 66, 82, 128). The three major control areas are monitoring of brewers yeast quality; detection, enumeration, and control of microbial contaminants; and sanitation of the brewing environment.

Yeast quality can be determined by periodic measurement of the following parameters during the course of fermentation: fermentation rate (226), flocculation characteristics (41), degree of wort attenuation (210), total number of cells and percentage of budding and viable cells (122), chain length of cells (26), effect on beer flavor, and the presence or absence of foreign microorganisms. Some of the more recent innovations include the use of fluorescent dyes (74) in place of methylene blue or rhodamin B (177) to determine yeast cell viability, and the use of a Coulter counter in place of a hemocytometer to determine cell numbers (144).

The most reliable method for detecting contaminant organisms is plating on a differential growth medium (75, 119, 127, 169). Direct microscopic examination is often of little or no value since the contamination level may be too low to detect in this manner (20).

Recent years have seen marked improvement in formulation of media for detection of microbial contaminants due largely to a better understanding of their exact nutritional requirements (21). An excellent example is Nakagawa's medium (169) for detection of beer sarcinae. It contains 1. mannose, an energy source not used by lactobacilli, 2. ascorbic acid for inhibition of aerobic bacteria, and 3. actidione to inhibit brewers' yeast. A low pH serves to inhibit acid-sensitive bacteria.

Two additional measures used by many breweries to offset yeast contamination are acid washing of harvested yeast (see section on Yeast Management) and periodic replacement of yeast with a fresh culture (see section on Yeast Propagation). Fresh cultures are also employed to help correct inherent yeast differences such as a reduced rate of fermentation which may be the result of a genetic change in the yeast population.

One of the greatest deterrants to microbial contamination of brewers yeast is in-place cleaning (Editorial. 1969. *Intern. Brewers J.* 105:1251:59–67) which, in recent years, has helped revolutionize brewing quality control. As indicated earlier, the success of such an operation is dependent on having a closed system from kettle boil through fermentation. By definition, in-place cleaning is the process of circulating cleaning solutions through process equipment and removing the soiling material by chemical action. Two types of circuits are in use, a closed circuit for heat exchangers and pipe lines and an open circuit for brewhouse vessels, fermentors, and the like. Automation is possible and seepage of cleaning solution past a damaged valve seating is eliminated by arranging for the

product to maintain a higher pressure than the cleaning solution. A wide choice of cleaning and sanitizing agents is available, the proper choice depending in part on the nature of the material to be cleaned (180). Some of the more commonly used sanitizing agents include various halogens (76), quaternary ammonium compounds (90), and miscellaneous compounds such as formaldehyde (167) and chloramine T (8).

Sanitation of the brewery environment is achieved in part by the following: (*a*) the use of ultraviolet light for sterilization of water used to rinse equipment and lines. The ultraviolet light source may be incorporated directly in the city water line. In order to achieve 100 per cent sterilization, it is important that the water be free of foreign particles such as iron deposits which can shield bacteria from the light source. (Kleyn, J. 1963. Personal observation). (*b*) Ultraviolet light may similarly be used for air sterilization (126). However, it is not thought to be in wide use with the possible exception of aseptic filling room sterilization. (*c*) Dehumidification of various cellars and packaging areas, thereby inhibiting mold growth which, in turn, will help eliminate musty odors as well as extend paint life (153). Passage of air through a dehumidification system also helps reduce the number of microorganisms present in the air. (*d*) Clean room techniques such as the use of positive air pressure in an aseptic filling room. Many of these are an offshoot of the space research program. (*e*) Incorporation of fungicides into grouting cement used in fermentation cellars.

ASEPTIC PACKAGING

Aseptic packaging is a brewing process whereby a biologically stable package beer is produced by means other than conventional tunnel pasteurization. Current commercial practices include flash pasteurization, membrane filtration, and chemical additives. Other proposed methods include ionizing radiations and high-frequency electrical fields (138). A major advantage relates to producing beer with an improved draft beer-like flavor. Conversely, there are brewers who will not adopt this process since tunnel pasteurization is believed to make a positive contribution to the flavor of their beer. A second advantage in certain aseptic packaging methods is a reduced cost of packaging as compared to conventional tunnel pasteurization (19). A third advantage relates to a saving of space through the elimination of conventional tunnel pasteurizers which require considerable floor area. For a recent review on aseptic packaging consult Portno (187).

One problem posed by flash pasteurization and membrane filtration and, to some extent, by chemical additives is the necessity of having an essentially sterile packaging area coupled with brewery workers trained in microbiological techniques necessary for aseptic packaging. Additional requirements to facilitate aseptic packaging include: (*a*) production of a beer with little fermentable sugar present. Absence thereof will inhibit reproduction of most contaminant microorganisms. The desired attenuation can be achieved through use of the proper yeast strain coupled with good brewing

and fermentation practices. (*b*) Production of beer with a low oxygen content. Oxygen stimulates both microbial growth and oxidative rancidity and should therefore be reduced to a minimum. Methods proposed for reducing the air content of beer include the use of nitrogen in various stages of the bottling process (99). (*c*) Production of beer with a low number of organisms able to grow in packaged beer. A major requirement herein is selection of a brewers yeast strain which is unable to grow in well-attenuated beer. If microbial contaminants are present in high numbers before filtration, one will not be able to eliminate them with filtration (172). High numbers also pose a problem with chemical additives since their activity is dependent in part on the concentration of contaminant microorganisms present in the beer.

One of the major contaminants found in spoiled package beer is *Saccharomyces diastaticus* (77, 124, 219), a yeast able to utilize dextrins left in beer which regular brewers yeasts, *S. carlsbergensis* and *S. cerevisiae* cannot degrade (70). Greenspan (77) determined that as few as four *S. diastaticus* cells could infect a package beer of any size. Brumsted & Glenister (28) determined that a second major contaminant of package beer, a *Lactobacillus,* could infect with as few as one cell per 12 oz. bottle of beer. Both of the above organisms grow well in package beer in that they can tolerate reduced oxygen concentrations. Other organisms found occasionally in spoiled package beer include members of the genus *Pediococcus, Obesumbacterium,* and *Brettanomyces.*

Major problems of flash pasteurization include defects in beer flavor due in part to uneven heating of the beer and the requirement for aseptic filling. The latter requirement is also necessary when using membrane filtration but is avoided to some extent by chemical additives. Mulvany (168) and Posada & Galindo (181) compare merits and demerits of the first two mentioned processes. Chemical additives currently used in beer include *n*-heptyl *p*-hydroxybenzoate (216) and octyl gallate (140). Both compounds, although effective, have their limitations, thereby providing opportunity for new and better chemical additives. Some other preservatives evaluated but not in use commercially include diethyl pyrocarbonate (166), hydrogen peroxide (29), and salts of ethylene diamine tetraacetic acid (121).

The mechanism of action of *n*-heptyl *p*-hydroxybenzoate is thought to involve destruction of the cytoplasmic membrane (137), whereas EDTA is thought to function by binding metal ions such as Mg^{++} essential for yeast growth (84). Interestingly enough, the activity of *n*-heptyl-*p*-hydroxybenzoate is enhanced by the addition of Ca^{++} or Mg^{++} (137), whereas the reverse occurs with EDTA (121).

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