

FERMENTED AND ACIDIFIED VEGETABLES

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50.1 INTRODUCTION

Vegetables may be preserved by fermentation, direct acidification, or a combination of these along with other processing conditions and additives to yield products that are referred to as pickles. Pasteurization and refrigeration are used to assure stability of certain of these products. Organic acids and salt (sodium chloride) are primary preservatives for most types of pickles. Lactic acid is produced naturally in fermented products. Acetic acid (or vinegar) is the usual acid added to pasteurized, unfermented (fresh-pack) pickles. Acetic acid also is added to many products made from fermented (saltstock) cucumbers. Other preservatives such as sodium benzoate, potassium sorbate, and sulfur dioxide may be added to finished products. Although the term "pickles" in the United States generally refers to pickled cucumbers, the term is used herein in a broader sense to refer to all vegetables that are preserved by fermentation or direct acidification. Cucumbers, cabbage, olives, and peppers account for the largest volume of vegetables and fruits commercially pickled. Lesser quantities of onions, tomatoes, cauliflower, carrots, melon rinds, okra, artichokes, beans, and other produce also are pickled.

The fermentation of vegetables is due primarily to the lactic acid bacteria, although yeasts and other microorganisms may be involved, depending on the salt concentration and other factors. Salt serves two primary roles in the preservation of fermented vegetables: It influences the type and extent of

Revised from earlier editions, includes some information previously provided by J. L. Etchells (deceased) and T. A. Bell (retired).

microbial activity, and it helps prevent softening of the vegetable tissue. Some vegetables are brined at such high salt concentrations as to greatly retard or preclude fermentation. Salt may be added in the dry form, as with cabbage, or as a brine solution, as with most other vegetables. The concentration of salt used varies widely among vegetables, depending on tendency of the vegetable to soften during brine storage. Softening of brined cucumbers can be reduced or prevented by adjusting the level of salt to inhibit pectinolytic enzymes.^{2,3} Fermentation is an economical means for temporary preservation of produce such as cucumbers, cabbage, and olives. The produce is fermented and stored in large tanks until it is needed for further processing. After removal from brine storage, brined cucumbers may be desalted if needed before being finished into various products such as dills, sweets, sours, hamburger dill chips, mixed vegetables, and relishes.⁴² Finished saltstock dill cucumber pickles contain a minimum of 0.6% lactic acid, according to USDA grade standards.¹⁰⁶ The products may or may not be pasteurized, depending on the addition of sugar and other preservatives. Extensive reviews are available on the brining and fermentation of cabbage,^{92, 101} cucumbers,^{40, 45} and olives.^{44, 108, 109, 110}

Direct acidification with acetic acid (without pasteurization) has been a primary method for many years of preserving various pickles and sauces in the United Kingdom, where the products are referred to as acetic acid preserves. British researchers have determined that the minimum acetic acid concentration necessary to achieve satisfactory preservation of all pickles and sauces is 3.6%, calculated as a percentage of the volatile constituents of the product.⁷ The high concentration of acid needed for preservation results in such a strong acid flavor, however, that the relative importance of this method of preservation has diminished. Milder acidic flavors are more in demand today, and use of acidification in combination with pasteurization has become more important. Nevertheless, some specialty products such as hot pepper sauce and sliced peppers still are preserved principally by high concentrations of acetic acid without pasteurization.

Fresh-pack cucumber pickles are preserved by mild acidification (0.5% to 1.1% acetic acid¹⁰⁶) of fresh cucumbers, followed by heating to an internal product temperature of 74°C and holding for 15 min, according to the original recommendations of Etchells et al.^{24, 30, 85} Such products are effectively pasteurized, since they are heated enough to inactivate microbial vegetative cells, and sufficient acid has been added to prevent outgrowth of bacterial spores. Although some packers still use this heat process, others now vary the times and temperatures, depending on product type and risk factors (not health-related spoilage) acceptable to the packer. Fermented pickles, such as whole genuine dills and hamburger dill chips, may or may not be heated. If pasteurized, these products may be given a milder heat treatment than fresh-pack pickles, such as an internal product temperature of 71°C with no holding time. The fresh-pack process has been applied to peppers and other vegetables. Fresh-pack pickles are considered acidified foods for regulatory pur-

poses. According to the U.S. Food and Drug Administration (FDA), " 'Acidified foods' means low-acid foods to which acid(s) or acid food(s) are added; these foods include, but are not limited to beans, cucumbers, cabbage, artichokes, cauliflower, puddings, peppers, tropical fruits, and fish, singly or in any combination. They have a water activity (α_w) greater than 0.85 and have a finished equilibrium pH of 4.6 or below. These foods may be called, or may purport to be, 'pickles' or 'pickled . . . ' "107

Refrigerated pickles may or may not be fermented before refrigeration. Also, they may or may not be acidified, although mild acidification is highly recommended.⁴¹ Most commercially prepared and distributed refrigerated pickles sold today are not fermented, but are acidified and contain a preservative such as sodium benzoate.

Increasing environmental concerns related to waste disposal are influencing methods for preservation of pickled vegetables, particularly those involving use of salt for bulk storage. The U.S. Environmental Protection Agency (EPA) has proposed a maximum of 230 ppm of chloride in fresh waters,²³ a limit that may not be readily achievable by many vegetable briners who discharge chloride wastes into freshwater streams. Organic acids (lactic and acetic) in combination with calcium chloride and preservatives (e.g., sodium benzoate) are now used instead of sodium chloride for bulk storage of olives for "green-ripe" processing into canned black olives in California.¹¹¹ Salt is still used, however, for fermented olives. The use of calcium salts (chloride or acetate) has led to reduced levels of sodium chloride for bulk fermentation and storage of cucumbers. Calcium salts have been found to enhance firmness retention of cucumbers at reduced concentrations of sodium chloride.^{10, 53, 56, 69, 105} Recent studies have revealed, however, that spoilage microorganisms may present a serious problem in fermented cucumbers if the salt concentration is too low.⁵⁹

50.2 NORMAL FLORA

Fresh produce contains a varied epiphytic microflora (Chapter 49). Pickling cucumbers were found to contain as high as 5.3×10^7 total aerobes, 1.9×10^4 aerobic spores, 9.8×10^5 total anaerobes, 5.4×10^2 anaerobic spores, 6.1×10^6 coliforms, 5.1×10^4 total acid formers, 4.6×10^3 molds, and 6.6×10^3 yeasts per g of fresh cucumber.³⁸ The numbers increased during storage at higher temperatures (21°C) and humidity (> 70% relative humidity). Although some investigators have held that the interior of sound, fresh cucumbers is sterile, others have found microorganisms, mostly gram-negative rods, within the healthy fruit.^{82, 97} In cucumbers, bacteria were more often near the skin and less often in the central core; in tomatoes, their frequency was highest near the stem-scar and central core and decreased toward the skin.⁹⁷ Cabbage contains the greatest number of bacteria on the outer leaves and lower numbers toward the center of the head.⁹²

The floral changes during natural fermentation of brined vegetables may

be characterized into four stages: initiation, primary fermentation, secondary fermentation, and post-fermentation.⁴⁵ During initiation, the various gram-positive and gram-negative bacteria that were on the fresh vegetable compete for predominance. *Enterobacteriaceae*, aerobic spore-formers, lactic acid bacteria, and other bacteria may be active. Eventually, the lactic acid bacteria gain predominance by lowering the pH, and primary lactic fermentation occurs. During primary fermentation, five species of lactic acid-producing bacteria are active, listed in approximate order of their occurrence: *Streptococcus faecalis*, *Leuconostoc mesenteroides*, *Pediococcus cerevisiae* (probably *P. pentosaceus* and/or *P. acidilactici*, according to recent classification¹⁰⁰), *Lactobacillus brevis*, and *Lactobacillus plantarum*. Although all five species are active during fermentation of sauerkraut,⁹² which contains relatively low concentrations of salt (c. 2.25%), only the latter three species predominate in fermentation of cucumbers, which contain higher concentrations of salt (c. 5% to 8%).⁴⁰ *Lactobacillus plantarum* characteristically terminates the lactic fermentation, apparently because of its greater acid tolerance.⁹¹

During fermentation of brined cucumbers, lactic acid bacteria may grow within the cucumber tissue as well as the brine.¹⁶ Gas composition of the cucumbers at the time of brining greatly influences the ratio of bacterial growth in the cucumbers and the brine.¹⁹ Yeasts were found not to grow within the cucumber tissue, presumably because of their larger size, which prevented their entry through stomata of the cucumber skin.

Green olives contain inhibitors of lactic acid bacteria,^{47, 49, 71} which are thought to influence fermentation of Spanish-type green olives.^{36, 72} Yeasts are not inhibited and predominate in the fermentation when the olives are neither properly lye treated nor heat shocked before brining.³⁶

Various species of fermentative yeasts also are active during primary fermentation. If fermentable sugars remain after primary fermentation, these sugars may give rise to secondary fermentation dominated essentially by yeasts. Fermentative yeasts grow as long as fermentable sugars are available; this may result in severe gaseous spoilage (bloat formation).^{26, 33, 32} During post-fermentation, growth of oxidative yeasts, molds, and bacteria may occur on brine surfaces of open tanks that are not exposed to ultraviolet radiation of sunlight.^{27, 88} Vegetable brining tanks are typically uncovered and are held outdoors to allow sunlight to reduce or prevent surface growth. Surface growth does not occur in fermented and anaerobically stored green olives.¹⁰⁹ Attempts are being made to develop a suitable anaerobic tank for the cucumber-brining industry.

Attempts have been made to use lactic starter cultures in sauerkraut, olives, cucumbers, and other products.⁴⁶ *Pediococcus cerevisiae* and *L. plantarum* have been used in pure culture or controlled fermentations of cucumbers^{35, 39} and olives.³⁶ Although starter cultures have been used on a limited commercial scale for fermenting cucumbers over the past 10 years, they are not widely used.

50.3 FLORA CHANGES IN SPOILAGE

50.31 Saltstock Vegetables and Genuine Dill Pickles

Vigorous activity in the cover brine by coliform bacteria, obligate halophiles, heterofermentative lactic acid bacteria, and fermentative species of yeasts is associated with gaseous fermentation and resulting bloater spoilage. Even homofermentative lactic acid bacteria such as *L. plantarum* and *P. cerevisiae* produce sufficient CO₂, when combined with CO₂ from cucumber tissue, to cause bloater formation in brined cucumbers.⁴⁸ Recent studies have shown that the major source of CO₂ production by homofermentative lactic acid bacteria is decarboxylation of malic acid, a natural constituent of pickling cucumbers.⁷⁹ It has been demonstrated that cultures that do not degrade malic acid ferment cucumbers with reduced bloater damage.⁸⁰ Procedures have been developed to produce and isolate nonmalate-decarboxylating mutants of *L. plantarum*.^{18, 20} Purging fermenting cucumber brines with nitrogen has been shown to be effective in preventing bloater formation.^{14, 39, 48, 52} Purging is now widely used by the pickle industry. Air purging also is effective in preventing bloater formation,^{14, 52} but can result in cucumber softening due to mold growth,^{15, 52, 62} reduced brine acidity due to yeast growth,⁹⁴ and off-colors and -flavors unless the purging regimen is carefully controlled. Bloater formation has been attributed to growth of gas-forming microorganisms in the brine surrounding the cucumbers³⁷ or within the cucumber.^{16, 96}

Softening of brined vegetables is caused by pectinolytic enzymes of plant or microbial origin. Growth of film yeasts on brine surfaces may occur and result in loss of brine acidity. Accompanying mold growth on the brine surface can cause softening of sauerkraut, cucumbers, or olives. Heavy scum yeast and/or mold growth is usually the result of neglecting brined material during the curing and storage period. Softening of brined cucumbers may result from mold polygalacturonases that accompany the cucumbers, especially cucumbers with flowers attached,^{5, 34} into the brine tank. This problem may be reduced by draining and rebrining of the tank c. 36 hr after initial brining. Recycled brine may be treated to inactivate softening enzymes.^{63, 78} Adding calcium chloride can slow down the rate of enzymatic softening of fermenting cucumbers.¹¹ However, this should not be relied upon to eliminate enzymatic softening problems. Care must be taken to minimize contamination of cucumbers, particularly the small fruit, with flowers and plant debris, which can be a source of contamination by pectinolytic molds.

Butyric acid spoilage of brined olives has been attributed to two distinct types of microbial action. In one type, *Clostridium butyricum* and a closely related group of clostridia produce butyric acid from sugars during the primary stage of fermentation.⁶⁵ In a second type of malodorous olive fermentation, "zapatera" spoilage results from decomposition of organic acids at a time when little or no sugar is present and the lactic acid fermentation stops before the pH has decreased below pH 4.5.⁷³ Propionibacteria were isolated from

brined olives with indications of "zapatera" spoilage and were hypothesized to grow and cause a rise in pH because of degradation of lactic acid, thus permitting subsequent growth by *Clostridium* species.^{8, 67, 93}

Recently, butyric acid spoilage of brined cucumbers was found to occur after an apparently normal primary fermentation by lactic acid bacteria.⁵⁹ *Clostridium tertium* was identified as contributing to the spoilage. Evidence from end products indicated that unidentified bacteria, possibly propionibacteria species, degraded lactic acid, causing a rise in pH that allowed *C. tertium* to grow.

50.32 Finished Pickle Products from Saltstock Vegetables (Not Pasteurized)

Fully cured saltstock vegetables are made into various types of finished pickle products by a series of operations involving leaching out most of the salt, souring with vinegar, and then sweetening with sugar if desired. Preservation of these products depends on sufficient amounts of vinegar alone (for sour pickles), or a combination of vinegar and sugar with or without sodium benzoate (for sweet pickles).¹ If the concentration of these ingredients is inadequate and the product is not pasteurized, fermentation usually takes place. Osmotolerant yeasts are the principal spoilage organisms in such products.¹ Molds and film yeasts may grow on the surface of the liquid chiefly as the result of faulty jar closure. Lactic acid bacteria, propionibacteria and butyric acid bacteria also may cause spoilage in unpasteurized fermented vegetables that do not contain adequate concentrations of acetic acid or other preservatives.

50.33 Acetic Acid Preserves

The 3.6% acetic acid (as a percentage of volatile constituents) required for preservation of pickles and sauces⁷ is similar to, but slightly lower than the concentration found necessary by Bell and Etchells¹ to prevent yeast spoilage in finished pickles from saltstock cucumbers. Microbial spoilage apparently occurs in acetic acid preserves when the concentration of acid is marginal. Spoilage microorganisms include yeasts²¹ and lactobacilli, particularly the heterofermentative *Lactobacillus fructivorans*.²²

50.34 Pasteurized Pickle Products

Spoilage usually occurs in these products when they are improperly pasteurized or improperly acidified so that an equilibrated brine product of pH 3.8 to 4.0 is not achieved. Spoilage is due chiefly to acid-forming bacteria and, to a lesser extent, yeasts that survive faulty heat treatment, or butyric acid bacteria when the product is not acidified adequately at the outset. Molds and film yeasts are factors in cases of poor jar closure.

50.35 Refrigerated Pickle Products

1. Fermented

A wide array of fermented, refrigerated cucumber pickle products are prepared as specialty products.⁴¹ Examples of such products include overnight dills, half-sour dills, genuine kosher dills, kosher new dills, sour garlic pickles, half-sour new pickles, fresh-packed half-sour pickles, new half-sours, home-style new pickles, half-sour kosher new dills, and the like. The cover brine may or may not be acidified. The products are held in barrels for a few days or longer at room temperature and then refrigerated at 2° to 5°C. They may be distributed in bulk or in consumer-size glass containers. In some cases, they may be initially brined, held, and distributed in consumer-size containers. Under such conditions and at equilibrated brine strengths of 10 to 12 salometer (1 salometer = 0.264% salt by weight), microbial growth (chiefly coliforms, gas-forming and nongas-forming lactics, and fermentative yeasts) and enzymatic activity (pectinolytic and cellulolytic) together with the curing process continue at a slow rate.³⁷ Gaseous spoilage of the product is caused chiefly by the gas-forming microbial groups mentioned earlier. Gas production may be sufficient to reach 15 lb pressure within the container.

Softening problems may be even greater than for saltstock cucumbers since these products are held at much lower concentrations of salt. Fresh, whole garlic cloves and other spices are normally added to such products. These spices may contain high activities of softening enzymes that increase softening problems. In a few months, the stored pickles may have lost much of their characteristic flavor, texture, and color and also may be bloated because of gaseous fermentation by the principal gas-forming microbial groups present.

Whether these pickles are made in bulk or in the retail jar, the very nature of the product makes it difficult to maintain good quality for any reasonable length of time. The barreled product reaches the good manufacturing practices (GMP)-recommended brine pH of 4.6 or below for acidified foods, usually before refrigeration or shortly thereafter, and then slowly continues acid development. This recommended condition for brine-product pH cannot be assured for the product made in the retail jar because there is no uniform process accepted by packers wherein the product is acidified at the outset or where it is deliberately incubated for development of natural lactic acid fermentation.

Sauerkraut marketed in plastic bags in refrigerated display cases is preserved by the addition of sodium benzoate and bisulfite.¹⁰³ The shelf life of such products is influenced by chemical changes that may result in discoloration (browning) and objectionable flavor formation.

2. Not fermented

Most of these products for national distribution are acidified with vinegar to an equilibrium pH well below 4.6, contain 2% to 3% NaCl, and are immediately refrigerated upon packing.⁴¹ They may contain sodium benzoate

or other preservatives. Like the fermented refrigerated product, the cucumbers are not heated either before or after packing. If properly acidified, refrigerated, and preserved, the products will maintain acceptable quality for several months and do not present a public health concern. Recipes that do not contain vinegar or other acid in the initial cover liquor, however, should be viewed with great caution.

50.4 PATHOGENIC MICROORGANISMS

We know of no authenticated reports of pathogenic microorganisms associated with standard commercial pickle products prepared under "good manufacturing practices" of acid, salt, and sugar content (and combinations thereof) from brined, salted, and pickled vegetable brine-stock, including cucumbers. The Commissioner of the FDA stated that "No instances of illness as the result of contamination of commercially processed fermented foods with *Clostridium botulinum* have been reported in the United States."¹⁰⁷ Even so, certain types of microorganisms that may cause spoilage of the product may, at times, be encountered, such as molds, yeasts, and acid-tolerant lactic acid bacteria. These organisms, usually under conditions associated with neglect, may reduce the quality of the texture and flavor of the product—whether prepared in bulk or retail container—and render it unusable. However, these organisms are not considered human pathogens.

Essentially the same pattern of consumer safety applies to fresh-pack (pasteurized) pickle products. These products have continued to increase in popularity until they now use over 40% of the annual cucumber crop in the United States. These pickles usually are prepared from raw cucumbers, but may include other vegetables in a mixture; also, vegetables other than cucumbers may be packed, such as various types of peppers, okra, carrots, green beans, and tomatoes. The process calls for the packed product to be acidified at the outset with a sufficient amount of food-grade organic acid, e. g., vinegar, acetic acid, or lactic acid, to result in an equilibrated brine product pH of 4.0 or below (preferably 3.8). Vinegar (acetic acid) is usually the acidulant of industry choice for cucumber pickle products. The basic pasteurization procedure, with product heated to an internal temperature of 74°C and held for 15 min. has been used successfully by industry since c. 1940.^{24, 28, 30} Insufficient acidification of pasteurized pickles can result in butyric acid-type spoilage, possibly involving public health concerns.

Listeria monocytogenes, a foodborne pathogen, in recent years has become a major concern to the food industry. The bacterium is commonly found in the environment and has been isolated from various plant materials, including silage,⁴³ soybeans, corn,^{113, 114} and cabbage.⁹⁸ Beuchat et al.⁶ showed that *L. monocytogenes* was able to grow on raw cabbage and in cabbage juice. Conner et al.¹³ found death of *L. monocytogenes* (one strain tested, LCDC 81-861, is a pathogen isolated from coleslaw) to occur in cabbage juice adjusted to

pH \leq 4.6 with lactic acid and incubated at 30°C; at 5°C, the death rate was slower than at 30°C. However, two strains tested grew well at pH values of 5.0 to 6.1. In a nutrient medium acidified with hydrochloric acid, the minimum pH values at which growth of *L. monocytogenes* was detected at 30°, 20°, 10°, 7°, and 4°C were, respectively, 4.39, 4.39, 4.62, 4.62, and 5.23.⁶⁴ Johnson et al.⁷⁰ demonstrated that *Listeria* could be recovered from fermented sausage made with beef intentionally contaminated with the bacterium. To our knowledge, *Listeria* has not been reported in fermented vegetables. However, the observation that *Listeria* can be isolated from fermented materials (silage and sausage), coupled with the bacterium's ability to tolerate moderately low pH^{43, 64, 70} and high salt concentrations (growth in complex media at 10% salt⁹⁹) suggests that *Listeria* may pose a concern for mildly acidified or fermented vegetables.

50.5 RECOMMENDED METHODS

50.51 Collection and Storage of Brine Samples

In examination of pickle products, brine or pickle liquor covering the vegetable material is required. The size of container to be sampled may range from a small jar of pickles to a 1,000-bu tank of fermented brine stock. Brine samples from containers such as tanks and barrels should be taken for bacteriological analysis as follows:

Insert a suitable length of 3/16-in stainless steel tubing, sealed at one end with lead or solder and perforated with several 1/16-in holes for a distance of 6 to 8 in from the sealed end, through an opening between the wooden boards composing the false head down into the brine toward the mid-depth of the vegetable material. Withdraw brine through a sanitized, attached piece of rubber tubing into a 12-oz bottle. Fit the receiving bottle with a two-hole rubber stopper and two short lengths of glass tubing, one for the rubber tubing leading from the stainless steel sampling tube and the other for a suction bulb to start siphoning action. The length of the steel sampling tube is governed by the depth of the container to be sampled. Withdraw and discard approximately 24 oz of brine before taking the final sample, about 10 mL, into a sterile test tube. Sterile vacuum tubes with rubber stoppers are suitable. If microbial changes during the fermentation are to be followed, start sampling at the time the material is salted or brined and continue at regular intervals of 1 to 2 days during active fermentation. After sampling, wash the whole assembly thoroughly.

For tightly headed barrels such as those used for genuine dills and salted vegetables for nonpickle use, take the sample through the top or side bung. For smaller containers, such as jars or cans of pickle products, shake thoroughly and take the sample from the center of the material by means of a sterile pipette. Wash the tops of the metal cans with alcohol, flame, and

puncture. A beer can opener is useful for puncturing metal tops. If the containers show evidence of gas pressure, carefully release gas by puncturing the sanitized top with a flamed ice pick. Containers under heavy gas pressure may be refrigerated overnight to reduce the gas pressure prior to sampling.

Brine samples from actively fermented material should be examined as promptly as possible after collection to prevent changes in the microbial flora. The same is true for samples of packaged pickle products. If it is necessary to ship or store samples, this should be done under the best of refrigerated conditions; the elapsed time from collection to examination should not exceed 24 hr. When shipment by air is required, samples are collected in sterile, 16- \times 105-mm tubes fitted with plastic screw caps having rubber liners. Pulp and oil liners, or plastic liners such as teflon, may leak because of changes in air pressure.

Brine samples may be preserved for subsequent chemical determinations by the addition of one to two drops of toluene or Merthiolate (1% aqueous solution) per 10 mL of sample. Samples preserved with the above chemicals are unfit for human consumption and should be so marked.

Many techniques have been developed for sample preparation and storage of ascorbic acid samples. For fermented and acidified vegetables, quickly mixing a sample with at least four volumes of 3% wt/vol metaphosphoric acid is a good sample preparation procedure. Little or no ascorbic acid is lost after 24 hr of storage in the refrigerator. Metaphosphoric acid stabilizes ascorbic acid much better than sulfuric acid or oxalic acid does. To reduce dehydroascorbic acid to obtain the total ascorbic acid, 0.1% BAL (2,3-dimercapto 1-propanol) can be added to the metaphosphoric acid solution.⁶⁰ A recent review of ascorbic acid analysis of plant tissues is given by Helsper.⁶⁸

50.52 Microscopic Examination

Microscopic examination of brine and vegetable samples for bacteria and yeasts is helpful at times, particularly when carried out in conjunction with plate count observations.

1. Bacteria

Make direct counts for bacteria according to the following procedures:

Place 0.01 mL amounts of liquid on slides using a calibrated pipette or loop and spread evenly over a 1 cm² area; fix with heat.

Stain according to the Kopeloff and Cohen modification of the gram stain.⁷⁴ Count according to the Wang¹¹² modification of the Breed⁹ technique.

Report results as "numbers of different morphological types of gram-positive and gram-negative bacterial cells per mL of brine."

To determine the number of bacteria within brined vegetable tissue, blend the tissue to a homogeneous slurry and filter through coarse filter paper (Reeve Angel 202, Whatman Laboratory Products, Inc., Clifton, N.J.). Bacteria within the filtrate are then enumerated with a Petroff-Hausser counting chamber at a magnification of about 500 \times .¹⁹

2. Yeasts

Use the microscopic technique for determining yeast populations in fermenting vegetable brines and various types of finished pickle products undergoing gaseous spoilage by the organisms, particularly where populations are in excess of 10^4 cells per mL of sample and where yeast colonies are not required for isolation and study. The use of a vital stain permits differentiation of yeast population into viable and nonviable cells and increases the usefulness of the direct counting technique.

The counting procedure is essentially the method of Mills⁸⁴ as modified by Bell and Etchells¹ for counting yeasts in high salt content brines and in high sugar content liquors:

Add 1 mL of brine or pickle liquor sample to 1 mL of 1:5,000 (0.02%) erythrosin stain.

Shake the sample stain mixture to obtain an even suspension.

Using a 3-mm diameter platinum loop, transfer enough of the mixture to the area under the cover glass of an improved Neubauer double-ruled hemacytometer to fill the chamber in one operation.

Allow cells to settle for approximately 5 min, and count the yeast cells using a microscope equipped with a 4-mm objective and 15× oculars.

Record cells stained pink as "dead yeast cells" and unstained cells as "live yeast cells."

The number of yeast cells per mL of brine or pickle liquor may be calculated thus:

$$\frac{\text{Number of yeast cells counted} \times \text{dilutions} \times 250,000}{\text{Number of large squares counted}} = \text{Numbers per mL}$$

If only one side of the hemacytometer counting chamber is used (25 large squares), the lowest yeast count obtainable is 20,000 per mL, while if both sides are counted (50 large squares), a population as low as 10,000 per mL can be counted.

Report yeast count as "total yeast cells," "live yeast cells," and "dead yeast cells per mL of sample."

50.53 Titratable Acidity and pH

Determine titratable acidity of a 10-mL sample of the fermentation brine or finished pickle liquor (liquid of the final product) by diluting the sample with 30 to 50 mL of distilled water; titrate with 0.1 N NaOH using phenolphthalein as the indicator. Alternatively, samples may be titrated to pH 8.2 with a pH meter. Report values for fermented, brined samples as g of lactic acid per 100 mL of sample, and for finished pickle liquor samples as g of acetic acid per 100 mL of sample.

For a 10-mL sample, use the following calculations:

- a. mL of 0.1 N alkali used \times 0.090 = g of lactic acid per 100 mL.
- b. mL of 0.1 N alkali used \times 0.060 = g of acetic acid per 100 mL.

When only a small amount of the original sample is available, use a 2-mL amount for titration purposes. Such small samples are not recommended. For the 2-mL sample, multiply the mL of 0.1 N alkali by 5, then by the above number for lactic or acetic acid.

Carry out pH determinations of the samples with a pH meter, checking the instrument frequently with a standard buffer in the pH range of the sample under test.

50.54 Determining Chloride and Calcium Contents of Brine

It is often helpful to know the approximate salt content in performing microbiological examinations of brines. Use a salometer, and test about 200 mL of brine. A chemical test for salt is required for small amounts of sample or when a higher degree of accuracy is desired than that obtainable with the salometer.

The following method is recommended. Transfer 1 mL of sample to a flask, and dilute with 15 to 20 mL of distilled water. Titrate with 0.171 N silver nitrate solution, 29.063 g per liter, using 3 to 5 drops of 0.5% dichlorofluorescein as the indicator. Agitate to keep the precipitate broken up until a light salmon pink color develops. Report as "g of sodium chloride per 100 mL of the sample." When 1 mL of sample is titrated, each mL of silver nitrate solution is equal to 1 g of sodium chloride per 100 mL.

A rapid colorimetric procedure based upon calcium binding by methylthymol blue can be used to measure the calcium content of brines or blended tissue.⁶⁶ Samples are mixed with an equal volume of 4% wt/vol trichloroacetic acid solution. Acidified solution, containing 50 to 600 μg calcium, is added to a test tube, and reagent solution is added. An immediate absorbance change at 612 nm occurs. The relationship between calcium concentration and absorbance is hyperbolic. A standard curve can be constructed by hand, or the data may be fitted to a hyperbola using nonlinear regression.

50.55 Determining Fermentation Substrates and Products

To determine whether the intended fermentation occurred or to determine the nature of an off-fermentation, it is important to measure both the substrates and products of a fermentation process. High-performance liquid chromatography (HPLC) procedures to measure all major substrates and products of both heterolactic acid and homolactic acid fermentations have been developed using refractive index detection.⁸¹ Alternative HPLC columns can be used, depending on particular analytical goals and problems. We now use a calcium- rather than a lead-modified resin column for sugar determinations because the analysis time is shorter and fructose and ethanol coelute on the lead column. However, sucrose is stable during chromatography on the lead column, but unstable on the calcium column. Therefore, if sucrose is an important sugar in the samples of interest, the lead column is preferred. For

the analysis of organic acids, reversed-phase columns gradually lose resolution and need to be replaced periodically. Also, not all C_{18} columns adequately separate malic acid, lactic acid, acetic acid, and ethanol. Resin columns in the H^+ form are extremely stable and reproducible for organic acid analysis using 0.02 N sulfuric acid as the eluent. We have used a single column (Bio-Rad HPX-87H) for more than 3 years without loss of performance. One problem with this procedure for cucumber pickle analysis is that fructose and malic acid coelute. The coelution problem has been solved in two ways using the same column as cited above. Lázaro et al.⁷⁵ developed equations to quantify fructose and malic acid differentially based on peak heights obtained from ultraviolet and refractive index detectors connected in series. Frayne⁶¹ actually resolved malic acid and fructose by connecting two of the HPLC columns in series.

Though HPLC is today the method of choice for analysis of fermentations, it has the disadvantage that an expensive instrument is required. An alternative approach for analysis of many fermentation substrates and products is enzymatic analysis using commercially available kits. The analysis can be done manually with an inexpensive visible colorimeter or spectrophotometer. The main disadvantage is that only a single compound can be analyzed at a time. Compounds for which kits are available include glucose, fructose, malic acid, L-lactic acid, acetic acid, ethanol, and CO_2 . Commercial sources for such kits include Boehringer Mannheim Biochemicals and Sigma Chemical Company. Enzymatic analysis is the only routine way to measure the L-isomer of lactic acid specifically.

It is important for the stability of fermented vegetables that all fermentable sugars be metabolized by the end of the fermentation process. For products like cucumbers that have little or no sucrose, colorimetric measurement of reducing sugars is a simple, rapid quality control procedure to assess the completion of sugar utilization. The dinitrosalicylic acid (DNS) procedure^{83, 104} is recommended. The reagent is stable at room temperature for many months. The assay can be reliably performed with an inexpensive colorimeter. A fermentation can be considered complete if the brine contains less than 0.05% reducing sugar and acid shows no increase for several days.

50.56 Determining Softening Enzyme Activity

Softening enzymes in brines of fermenting cucumbers and other vegetables may be determined by the highly sensitive viscometric method of Bell et al.⁴ The procedure, which has been widely used in the pickle industry for many years, is based on viscosity loss of a buffered polypectate solution. Brine samples, 25 mL, are dialyzed in running water for 3 hr and distilled water for 1 hr. One mL of the dialyzed sample is added to 5 mL of 1.2% sodium polypectate, which is dissolved in 0.018 M, pH 5.0 citrate buffer in an Ostwald-Fenske no. 300 viscometer. A drop of toluene is added to the sample to

prevent microbial growth during incubation. The flow time of the pectate solution is measured after sample addition and at 20 hr. The viscosity loss is calculated according to the following equation:

$$\text{Percent loss in viscosity} = \frac{A - B}{A - W} \times 100, \quad (1)$$

where A is the initial flow time in seconds, B is the flow time at 20 hr, and W is the flow time for water. Bell et al.⁴ provide a table that relates loss in viscosity to the units of pectate depolymerizing activity. A less than 9% loss of viscosity in 20 hr is considered to represent weak to negative activity in brine samples.

Refer to Chapter 14 for isolation of pectinolytic organisms and characterization of pectinolytic enzymes.

50.57 Determining Dissolved Carbon Dioxide

The advent of purging to remove CO_2 from fermenting cucumber brines and thus prevent bloater formation has created a need to determine the concentration of dissolved CO_2 in the brine. For the highly accurate determinations that may be required for research purposes, dissolved CO_2 is determined by the micro distillation procedure.⁵¹ A 10-mL brine sample is injected by syringe into a capped jar containing an acid solution. A small vial of standardized NaOH placed inside the jar traps the CO_2 as it distills from the acidified solution. After 24 hr at 37°C , the vial is removed, BaCl_2 is added, and the remaining base is titrated to the phenolphthalein end-point with HCl . Values are expressed as mg CO_2 per 100 mL brine.

For quick estimates that may be required for quality control tank monitoring, dissolved CO_2 is determined with a micro CO_2 apparatus. Adaptation of this instrument for the determination of CO_2 in fermenting cucumber brines has been described.⁵⁰ This is a gasometric method based on the classical Van Slyke procedure. A 1-mL brine sample is placed in the instrument vial, a volumetric syringe is clamped into place, an acid solution is added, the apparatus and sample vial are shaken, and the gas volume displacement is read on the calibrated syringe scale. Carbon dioxide in the brine sample is calculated from scale readings of the brine compared to a CO_2 solution of known concentration and is expressed as mg CO_2 per 100 mL brine. It is suggested that brine samples be taken from brine tanks through a siphon tube (see Section 50.51) and 8.5 mL syringed through a needle into a Vacutainer tube (10 mL draw, Becton-Dickinson, containing 0.5 mL of c. 3 N NaOH) to minimize CO_2 loss. The samples are then equilibrated to the same temperature as the known solution before analysis.

In both methods, the total CO_2 content of the solution is determined and is expressed as mg CO_2 per 100 mL brine, or as percent saturation.⁵⁴

50.58 Microbiological Analyses

1. Aerobic plate count

Use plate count agar or nutrient agar and incubate for 3 days at 32°C. Overlay the solidified, plated samples with about 8 to 10 mL of the same medium to prevent or minimize spreaders.

2. Lactic acid bacteria

Lactic acid bacteria associated with pickled vegetables of the genera *Lactobacillus* and *Pediococcus* are selectively enumerated with *Lactobacillus* selection medium (LBS), appropriately modified as noted below. Overlay the plated samples with the same medium to permit earlier enumeration of colonies. Incubate at 32°C for about 4 days or until suitable colony enlargement occurs. The incubator should be humidified to retard desiccation of the medium during incubation. Fructose, 1%, may be added to the medium to ensure greater enumeration of certain lactobacilli.¹⁰² Bromocresol green (or brilliant green, as in Chapter 62), 0.0075%, may be added to aid in colony counting, but may further retard growth of lactic acid bacteria in an already inhibitory medium. Cycloheximide, 200 ppm, should be added as needed to inhibit yeasts.

... Total lactic acid bacteria may be estimated by plating samples in MRS agar containing 0.02% sodium azide and incubating for 1 to 4 days at 30°C.¹⁷

To enumerate differentially all species of lactic acid bacteria associated with vegetable fermentations, plate fermenting samples in a noninhibitory medium such as tryptone-glucose-yeast extract agar.⁹⁰ After incubation at 32°C for 48 hr, isolate colonies for later identification on the basis of acid and gas production, cell morphology, and mucoid growth;⁸⁹ other reactions may be used.

The decarboxylation of malic acid to lactic acid and CO₂ is an undesirable reaction in cucumber fermentations because of the CO₂ produced. Most lactic acid bacteria can decarboxylate the malic acid in cucumbers. Specific strains of *L. plantarum* have been developed that no longer can decarboxylate malic acid.¹⁸ To distinguish between malate-decarboxylating (MDC⁺) and malate-nondecarboxylating (MDC⁻) lactic acid bacteria, a differential medium has been formulated (MD medium, see Chapter 62 for composition), which is suitable as either a broth or a plating medium. The differential reaction is based upon pH changes in the medium caused by malate decarboxylation. A pH decline (MDC⁻) is shown by a color change from blue to green, whereas there is no color change for the MDC⁺ reaction. This medium is not selective for lactic acid bacteria.

HHB medium (see Chapter 62 for composition) is used for the differential enumeration of homofermentative and heterofermentative lactic acid bacteria.⁷⁶ This medium incorporates fructose, which is reduced to mannitol by heterofermentative but not homofermentative lactic acid bacteria. In agar medium, homofermentative colonies of lactic acid bacteria are blue to green,

while heterofermentative are white. HHD medium is not selective for lactic acid bacteria.

3. Total *Enterobacteriaceae*

Add 1% glucose to violet red bile agar, which is referred to as MacConkey glucose agar.⁸⁶ Incubate for 24 to 48 hr at 32°C.

4. Coliform bacteria

Use violet red bile agar, incubate plates at 32° or 35°C for 24 hr, and count all purplish red colonies surrounded by a reddish zone of precipitated bile, 0.5 mm in diameter or larger.

5. Yeasts and molds

Acidify sterile, tempered, 45°C dextrose agar with 10% tartaric acid (usually 5% by volume) to achieve a final pH 3.5 ± 0.1 . Potato dextrose or malt agar may be used when acidified to pH 3.5 as above. Incubate for 3 to 5 days at 30°C.

Alternatively, the antibiotic method described in Chapter 16 may be used. Small colonies of lactic acid bacteria may appear on the acidified medium above, but are suppressed in the antibiotic medium. We use plate count agar (Chapter 62), supplemented with a solution containing 500 mg each of chlor-tetracycline HCl and chloramphenicol in 100 mL of sterile, distilled water and added aseptically to the tempered agar (45°C) at a rate of 2 mL of antibiotic solution per 100 mL medium, as recommended in Chapter 16.

Mold colonies are filamentous and, thus, are distinguished readily from yeasts on acidified dextrose agar. Differentiation of subsurface yeasts and film yeasts presents more difficulty. Surface colonies of the common film-forming yeasts associated with pickle products and vegetable brines, i.e., species of *Debaryomyces*, *Endomycopsis*, *Candida*, and *Pichia*,^{27, 87} are generally dull and very rough, as contrasted to the usual round, raised, white, glistening colonies of the fermentative, subsurface yeasts, i.e., species of *Torulopsis*, *Brettanomyces*, *Hansenula*, *Saccharomyces*, and *Torulasporea*.^{29, 32, 33} However, even when distinguishing colony characteristics of the two yeast groups exist, they are not considered sufficiently clear-cut for separation. Because of this, the procedure outlined under Section 50.58.6 should be used. Film yeasts rapidly form a heavy wrinkled surface film at one or both salt concentrations. Certain species, such as *Saccharomyces halomembranis*, form heavier films at 10% salt than at 5%.^{25, 27, 31, 87}

6. Film yeasts

For an estimate, pick representative filamentous colonies from the yeast plates into tubes of dextrose broth containing 5% and 10% salt. Incubate 3 to 5 days at 32°C and observe for heavy surface film. Two salt concentrations are suggested because some species develop heavier films at the lower salt strength (5%), whereas, with other species, the reverse is true.

7. Obligate halophiles

Use tubes of liver broth plus salt (Chapter 62). Prepare decimal dilutions, seal with sterilized, melted petroleum jelly, and incubate 7 days at 32°C. Record positive tubes daily by noting the raising of the petroleum seal caused by gas production and the absence of any distinctive odor.

This medium has proved satisfactory for detecting obligate halophiles sometimes found in brined and dry-salted vegetables. The salt content of the medium should approximate that of the sample. No growth of coliforms or yeasts has been encountered in this medium. This is probably due to the inability of either group to initiate satisfactory early growth in laboratory media even at moderately high salt concentrations in competition with the very fast-growing obligate halophiles.

8. Butyric acid-forming bacteria

Neutralize the brine sample with an excess of sterile calcium carbonate. Heat a 50- to 100-mL sample in a waterbath for 20 min at 80°C to kill vegetative cells. Prepare decimal dilutions and inoculate previously heated and cooled tubes of liver broth medium. Seal with melted petroleum jelly and incubate 7 days at 32°C. Examine tubes daily for production of gas and a strong butyric acid odor.

50.6 INTERPRETATION OF DATA

50.61 Saltstock Vegetables and Genuine Dill Pickles

Proper record-keeping of salting procedures and chemical and microbiological data can greatly aid the commercial briner in assessing causes for success or failure in preserving the quality of brined vegetables. Records of chemical determinations of salt, titratable acidity, pH, fermentable sugars, dissolved CO₂, and softening enzyme activity are very useful in such assessments, depending on the particular commodity.

In fermented vegetables, it is important that the lactic acid fermentation become established early to preclude growth by spoilage bacteria. Acidity and pH data provide this information. Salt concentrations above 8% for cucumbers and olives or above 2.5% for cabbage may prevent or retard a desirable lactic fermentation. Unusually low salt concentrations may result in softening of the brined vegetables.

If the dissolved CO₂ concentration in the brine of fermenting cucumbers is allowed to exceed about 50% saturation (equals 54 mg per 100 mL at 21°C and 6.6% NaCl) at any time during brine storage, bloater damage may result. Maintaining the brine CO₂ concentration below 50% saturation will greatly aid in reducing bloater damage.⁵⁴ Sporadic bloater damage may occur even in effectively purged brine-stock cucumbers. Such damage may be due to growth of bacteria within the brined fruit.¹⁶ Since brines must be purged as

long as fermentation occurs, it is important to monitor the level of fermentable sugars in the brine. When fermentable sugars are not detected and acid development has ceased, the fermentation is considered to be complete, and purging can be safely discontinued.

Microbial softening enzyme activity of brines may indicate the cause of soft brine-stock pickles, especially if the cucumbers are held at relatively low brine strengths (5% to 8% NaCl). Higher salt concentrations will prevent softening by these enzymes,² but high salt levels present disposal problems in addition to affecting the lactic fermentation adversely. Recent studies have indicated that calcium chloride, c. 0.2% to 0.4%, and other salts of calcium may inhibit the action of softening enzymes.¹⁰ Calcium chloride is now being added to commercial cucumber brines. The extent of protection against softening offered by calcium has not been fully assessed.

The absence of softening enzyme activity in older brine-stock pickles does not necessarily mean that such activity did not cause the softening. Softening enzymes that accompany the cucumbers and attached flowers into the brine tank may exert their influence early in brine storage and then be dissipated or inactivated so as not to be detectable later.

Softening in the seed area of large cucumbers, commonly termed "soft centers," is thought to be due to natural polygalacturonase of overly mature cucumbers,⁹⁵ not to microorganisms.

The advent of reliable HPLC procedures to measure changes in substrates and products of fermentations has made it practical to assess the balance between substrate utilization and product formation in complex food fermentations. Carbon recovery of <100% indicates that some fermentation products have been missed in the analysis, while recovery of >100% suggests that unknown substrates have been fermented. Examples of fermentation balances done on complex fermentations have been published.^{12, 55, 57, 58, 77} The determination of fermentation end products was extremely useful in recent efforts to determine microorganisms responsible for spoilage of fermented cucumbers.⁵⁹

50.62 Finished Pickle Products from Saltstock Vegetables

These products normally contain a few thousand microorganisms per mL. These counts may be composed chiefly of spores of aerobic bacteria that remain inactive in the acid medium and tend to decrease during storage. Fermentative yeasts and lactic acid bacteria may cause vigorous gas production, which causes the pickle liquor to become highly charged with gas and to possess a tang when tasted. Viable microorganisms, normally latent in properly fermented and preserved products, may cause gaseous spoilage in improperly finished products. Gaseous spoilage and cloudy cover brine may be the result in hamburger dill chips, genuine dill pickles, Spanish-style green olives, and similar products, if residual sugar remains.

50.63 Pasteurized Pickle Products

Properly acidified, packaged, and pasteurized pickle products are not subject to microbial spoilage. When spoilage occurs, it is usually due to under-pasteurization. Some commercial packers minimize heat processing in order to maintain greater product quality. Minimal processing is done at the risk of spoilage. Spoilage results in recall of the product at the packer's expense. No public health problem exists in pasteurized pickle products that have been properly acidified. After spoilage occurs, however, as evidenced by gas pressure and brine turbidity, there is no way to ensure that the product was properly acidified initially. Lactic acid bacteria are normally found in such products. The spoiled product usually contains acid, but it is not known at that point if the original product, particularly if it was fresh produce, was sufficiently acidified to prevent growth of *Clostridium* before growth of the lactic acid bacteria.

Improper acidification can also be a source of spoilage with potential public health significance, as discussed in Section 50.4. Improper closure can result in growth of aerobic microorganisms on the surface of the brine and a reduction in acidity.

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