Fermentation Microbiology

Making Cheese, Yogurt & Buttermilk as a Lab Exercise

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In the introductory microbiology laboratory, food microbiology exercises are frequently placed at the end of the quarter or semester, if time allows after covering medical bacteriology or mycology. In the instances when food microbiology is given attention earlier in the term, demonstrations are often used or discussed that still do not allow the students in the lab the chance to actually see what processes are taking place in, for example, a food fermentation setup. Although these topics could be presented in more detail in the lecture portion of the general microbiology class, the end results (products) of these fermentations are never seen by the students firsthand. The following exercises can be done by students in an introductory microbiology lab all at once or separately, as time allows, and result in fast, inexpensive and relatively foolproof food fermentation products that students can evaluate themselves—by eating! In each case, the procedure is presented first, followed by a discussion of the process and principles behind each fermentation reaction.

Exercise I—Cheese

This lab is performed using freeze-dried lactic acid bacteria (LAB) cultures purchased from the New England Cheesemaking Supply Company, PO Box 85, Ashfield, MA 01330; www.cheesemaking.com; tel: 413-628-3808 for catalog). Other related supplies can be obtained from this company, as well.

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Objective

Demonstrate proliferation and production of acid by LAB during the making of cheese.

Procedure

A. Take fresh pasteurized milk (usually 1 l) and warm to room temperature using a water bath or double boiler. Add the entire packet of cheese culture. Stir thoroughly and cover. Incubate at room temperature or in a 25° C incubator for 18 to 24 hours. When finished, the milk should be solid (coagulated); whey (pale yellow liquid) may be visible around the edges, and a clean acid aroma should be present.

B. Dump the coagulated milk into a colander lined with cheesecloth or coarse uncolored clean fabric and allow to drain for 4 to 6 hours. During this time it will be necessary to occasionally take a clean spoon and scrape the sides of the colander to facilitate whey draining, allowing the coagulated milk to become more solid and cream-cheese-like in appearance.

C. Dump the now mostly solid cheese into a bowl and sprinkle with salts and desired herbs (available from New England Cheesemaking Supply Co.) just for taste. Pack the cheese into a cheesemold (a teacup will do) and place in a refrigerator overnight. The next morning, free the cheese from the mold (it should be easy to remove). It is now ready to eat.

Discussion

What is milk? Milk is 88% water and 12% solids. Of these solids, 4% are fat, 3.5% are protein, 4% are lactose (milk sugar), and 0.5% are minerals.

What happened? The primary protein in milk is casein, which is sensitive to acid. In the presence of acid, caseins become less soluble and tend to clump together. Casein is also sensitive to a particular enzyme called rennet, an acid protease. This means that rennet acts on (cleaves) milk protein (casein) and has optimum activity under acidic conditions. When the enzyme rennet acts on casein, the latter becomes even less soluble. The loss of solubility is what makes the thick coagulum (curds) form in fermented milks. In this exercise the cheese culture that was added to the milk contained cheese LAB and powdered rennet. The bacteria produced lactic acid which lowered the pH and made casein less soluble. Under the acidic conditions produced by the LAB, the enzyme rennet cleaved the caseins and made them even less soluble, thus forming the coagulated milk which was then further drained to make cheese.

What is whey? Whey is the yellowish liquid that is produced during the making of fermented dairy foods. As the acid-sensitive caseins become less soluble and coagulate, water (whey) is squeezed out. If you have ever purchased sour cream, cottage cheese, or yogurt from the grocery store, the clear liquid that you sometimes see when you take off the lid is whey. Whey is mainly water, but it also contains small amounts of lactose, minerals and non-acid-sensitive milk proteins called ''whey proteins.''

Tremendous amounts of whey are produced during industrial cheesemaking. Because whey contains sugar and protein, it is an excellent growth medium for bacteria and has a high biochemical oxygen demand (BOD). Until several years ago, whey was

poured into the sewer system and was expensive for wastewater treatment facilities. Today, processes have been developed to re-process whey and use the sugar and proteins in it.

What are the bacteria used in cheese production? Cheese primarily uses genera of LAB called lactococci. In particular, *Lactococcus lactis* ssp. *lactis* and *Lactococcus lactis* ssp. *cremoris* are widely used to make cheddar cheese. (It should be noted here that the genus *Lactococcus* was created in 1985 to include the species formerly known as *Streptococcus lactis*. Although many general microbiology laboratory manuals still refer to this organism by its former name, the proper name is *Lactococcus lactis*). Lactococci are homofermentative, meaning the primary product from fermentation is lactic acid, and not a mixture of various organic acids (heterofermentative). Other types of cheese may employ other LAB to obtain different flavors.

Pasteurization is the process of heating milk so that all pathogenic bacteria are killed. Pasteurized milk is not sterile; this is why milk spoils after a few weeks in the refrigerator. However, no pathogens remain after pasteurization. The heat treatments used for pasteurization are defined by law: 63° C for 30 minutes or 72° C for 15 seconds. The latter heat treatment is often called High Temperature Short Time Pasteurization, or HTST. There are even higher heat treatments called Ultra High Temperature (UHT) treatments (usually 100° C for 1 to 2 seconds). All milk purchased in grocery stores is pasteurized.

Raw milk, however, can contain pathogenic bacteria and has been the cause of foodborne illness outbreaks. Pathogenic bacteria that can be in raw milk include *Escherichia coli* 0157:H7, *Listeria monocytogenes*, and *Staphylococcus aureus*. All of these bacteria are completely eliminated by pasteurization. However, not all of them are sensitive to acid, which allows them to survive during fermentation. A recent study in our laboratory has shown that *E. coli* 0157:H7 can survive (with LAB) for one month in buttermilk! For these reasons, *never* use raw milk for any of these experiments.

Exercise II—Yogurt

Procedure

A. Yogurt formula:

Milk (any fat content)	91%*
Nonfat dry milk powder	4%
Table sugar (sucrose)	5%

B. Preblend the powdered milk and sugar—no lumps!
C. Disperse the powdered ingredients into cold milk using a hand blender or regular blender.
D. Heat the mixture in a water bath to 85° C for 20 minutes.
E. Cool to 40° C in an ice bath. Add the powdered yogurt culture (available from New England Cheesemaking Supply Company) and stir thoroughly.
F. Incubate at 40° C for 8 to 16 hours (until a firm coagulum is formed). Yogurt can be cooled and consumed at this point. Fruit topping can be added to spruce up the taste.

*Total volume can vary, depending on class size, so the amounts here are expressed as percent figures, not in grams or milliliters. A 1-liter batch would implement, for example, 910 grams of milk, 4 grams of dry milk powder, and 5 grams of sucrose.

What happened? Yogurt is produced by bacterial fermentation. Unlike cheese production, rennet is not used. The thick consistency of yogurt is due to the decreased solubility of casein in the acid produced by the yogurt culture and by additional ingredients. Starch, pectin and gelatin are often added to commercial yogurt to give it a thick consistency. For our yogurt, powdered milk is added to provide this thicker consistency. Sugar is added to flavor the finished yogurt and to provide additional carbohydrate for the LAB. LAB can ferment lactose (milk sugar) and sucrose (table sugar).

Why is the yogurt mixture heated? Yogurt, unlike cheese, does not use the enzyme rennet to aid in gel formation. The heat treatment of 85° C for 20 minutes aids in the formation of a smooth gel (coagulum) by denaturing whey proteins present in the milk. Whey proteins are not present in high concentrations in milk (like casein) nor are they sensitive to acid (like casein). Whey proteins do, however, have a unique property. When subjected to moderately high temperatures, they denature, or unfold, which helps to trap water and forms a smooth coagulum. Buttermilk is heat treated for the same reasons.

What bacteria are used? All yogurt requires two bacteria: *Lactobacillus delbrueckii* ssp. *bulgaricus* and *Streptococcus thermophilus*. These two LAB together produce acid and typical yogurt flavor. Note that these two LAB are thermophilic, which is why the fermentation temperature for yogurt is much higher than the temperature used for cheese or buttermilk. Other bacteria are often added to yogurt for health reasons, particularly in the last few years. These bacteria are called probiotic bacteria, and they aid in digestion and intestinal health (but play no role in the production of yogurt). The two bacteria most commonly used for this purpose are *Lactobacillus acidophilus* and *Bifidobacterium longum*.

Exercise III—Buttermilk

Procedure

A. Buttermilk formula:

Milk (any fat content)	98%*
Nonfat dry milk powder	2%
NaCl	0.1%

B. Preblend the powdered milk—no lumps! Also add salt here, for flavor.
C. Disperse the powder into cold milk using a hand blender or regular blender.
D. Heat the mixture in a water bath to 85° C for 20 minutes.
E. Cool to 25° C in an ice bath; add the powdered buttermilk culture (from New England Cheesemaking Supply Co.). Stir thoroughly.
F. Incubate at 25° C for 16 to 24 hours (until a firm coagulum is formed). The buttermilk can now be cooled in a refrigerator and consumed.

*As for yogurt, the amounts here are expressed as percents, since volume can vary according to class size; the final percentage of each component here should stay consistent regardless of volume.

What happened? Buttermilk, like yogurt, does not use rennet, only LAB fermentation. The addition of powdered milk aids in a desirable thick texture. The heat treatment, as in yogurt, also facilitates a desirable texture.

What bacteria are used? Buttermilk uses species of *Lactococcus* and *Leuconostoc*. The lactococci are used for acid production and the leuconostocs for flavor. *Leuconostoc* spp. are able to produce diacetyl as a by-product of fermentation. Diacetyl gives high-quality buttermilk its characteristic delicate buttery aroma.

Exercise IV—Acid Production & Quantifying LAB

Materials

- pH meter
- Titrating apparatus (with 0.1N NaOH)
- Phenolphthalein indicator
- Litmus paper
- Trypticase soy agar (TSA)
- 99 ml sterile peptone blanks for dilutions
- Sterile plastic petri plates
- Sterile 1 ml disposable pipets or pipettors with sterile 1 ml tips

Background

A pH meter will measure the concentration of free hydrogen ions in a sample. Litmus paper is embedded with dyes that are sensitive to changes in hydrogen ion concentration; any color change can be related to a particular pH. Thus, a pH meter and litmus paper are essentially measuring the same thing. Titratable acidity (TA) measures both free and bound hydrogen ions. This is a measurement of acidity, as is pH, but is not exactly the same. Often used in addition to or instead of pH in the food industry, TA is very easy to determine and does not require expensive apparatus.

Procedure

Fill a buret with 0.1N sodium hydroxide. Weigh out 9 g of dairy product into a small beaker. Add to the beaker 2 drops of 1% phenolphthalein, made by dissolving 1 g of powdered phenolphthalein (Sigma, St. Louis, MO, P-9750) in 100 ml of 95% ethanol. (Phenolphthalein is a pH indicator.) Begin adding drops of sodium hydroxide from the buret to the beaker. Swirl the beaker to disperse the pink color that develops. When the pink color persists for 20 seconds or so, you have reached the end point of the titration. The solution in the beaker should be visibly faint pink. Read the amount of NaOH that has been used and record. Use the following formula to determine the percent of lactic acid or percent TA in the sample (from Marshall 1992):

$$\frac{(\text{ml NaOH used}) \times (\text{N NaOH}) \times 9}{\text{weight of sample, g}}$$

Typical TA values for yogurt are 1% to 2%, while the values for buttermilk are usually less than 1%. Regular milk will have a TA value of 0.14 to 0.16%. Note the increase in TA value upon lactic fermentation.

Changes in pH can also be monitored using a pH meter or litmus paper. Typical pH of regular milk is 6.4 to 6.6. The pH of cheese, yogurt and buttermilk is 4.0 to 4.8. So, changes in acidity before and after lactic fermentation can be demonstrated using one or all three of these methods.

In order to perform pour-plating to determine the number of viable bacteria in fermented dairy products, it will be necessary to melt the solid TSA in a boiling water bath (or equilibrate freshly autoclaved TSA in a 55° C water bath prior to using). In either case, do not pour the plates until the agar is equilibrated to 55° C or the bacteria will be killed.

A. Take 1 ml of buttermilk or yogurt (or 1 g if either is too thick to pipet) and place into a 99 ml dilution bottle containing sterile peptone. Cap tightly and shake gently 30 times in an up-and-down motion to disperse the sample.
B. From this dilution (10^{-2}), pipet 1 ml and place into another 99 ml peptone blank, cap and shake (total dilution now = 10^{-4}). Repeat once more to get a dilution of 10^{-6}.
C. Dispense 1 ml or 0.1 ml into sterile empty petri dishes to obtain total dilutions of 10^{-6} and 10^{-7} for an appropriate range of bacteria in fresh yogurt or buttermilk. Do duplicate plates for each dilution.
D. Pour equilibrated agar into the plates (enough to nearly cover the bottom of the plate) and gently swirl in a ''figure 8'' pattern on the benchtop to evenly disperse the bacteria. Let the agar solidify and pour another layer of molten agar on top of the first, approximately 0.5 cm thick (an overlay). Overlays are often needed for microaerophilic bacteria, such as LAB, to grow best.
E. Label the plates appropriately and incubate them for 24 to 48 hours at 32° C. Colonies will be observed as white or cream-colored specks in the agar. To calculate the number of bacteria per ml (or g) of dairy product, count the colonies on the plates. Select a dilution set containing between 30 and 300 colonies, count them, and determine the average of the two. Multiply this number by the inverse of the dilution. For example, an average of 120 colonies on the 10^{-6} dilution set would be 120 multiplied by 10^6, or 1.2×10^8 bacteria (sometimes called ''colony-forming units'') per ml or g of dairy sample.

Conclusions

This multi-part lab exercise can be fragmented to best suit the format of the class. The pH and pour-plating exercise can be teamed up with any or all of the fermentation portions to provide students with a straightforward and practical illustration of how dairy fermentations work, and how useful microbiological techniques like pour-plating are put to use in an industrial setting. Student response to these exercises has been very positive, because members of each class are naturally inquisitive about the food they eat. Instructors may wish to have students Gram stain some representative colonies on the last exercise to view LAB microscopically, or even try to cultivate these microorganisms on various other media to note differences in colonial morphology. Regardless, we feel that this set of exercises effectively demonstrates the unique properties of a lesser-studied but extremely useful group of bacteria—the lactic acid bacteria.

Reference

Marshall, R.T. (Ed.). (1992). *Standard Methods for the Examination of Dairy Products*, 16th ed. Washington, DC: American Public Health Association.