Microbiology of Cracker Sponge Fermentation[1]

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ABSTRACT

Lactic acid bacteria in commercial yeast and wheat flour were easily isolated from enriched cultures. The physical structure of dough (ie, limited water) impaired the physiological activities of the microflora, as shown by comparing plate counts of dough and of flour-water slurries. Pure culture yeast, added to dough, did not lower the pH below that of the indigenous microflora of the flour. Adding large numbers of lactic acid bacteria isolated from flour rapidly lowered the pH of the dough. Lactic acid bacteria were dominant in doughs and in flour-water slurries with added yeast. Possibly because of the competition for fermentable carbohydrate or the accumulation of toxic waste products, the number of yeast cells declined in dough and in flour-water slurries as a result of fermentation. Coliforms and proteolytic bacteria did not play a major role in the fermentation.

A sponge and dough process is used commercially to produce soda crackers. The 24-hr fermentation is thought to be responsible for crackers' unique flavor and textural properties. The chemistry (Heppner 1959, Matz 1968, Pieper 1971) and rheological changes in cracker sponges (Pizzinatto and Hoseney 1980) have been studied. Micka (1955) and Sugihara (1978a, 1978b) have also studied the microbiology of the process.

Because flour is the basic raw material in cracker dough, its examination as a source of, and a substrate for, microorganisms is important to our understanding of the microbiology of soda cracker dough fermentation. The flour reflects the microflora of the grain from which it is made. The numbers and types of microorganisms on any grain are related to many environmental factors such as rainfall, sunlight, temperature, season, birds, insects, rodents, and humidity (Hobbs and Greene 1976).

Milling reduces the bacterial load. Rogers and Hesseltine (1978) found that counts in 54 wheat samples varied from 870 to 3.1 $\times 10^6$/g and that counts for flour samples milled from those wheats were lower in every case.

All flours contain a microbial population consisting of fungi and bacteria (Hesseltine and Graves 1966); the microbial population varies in numbers, but the kinds of microorganisms are essentially constant. The most common species found in flour (Hesseltine and Graves 1966) and those isolated from refrigerator doughs and wheat flour (Rogers 1978) are: *Enterobacter aerogenes, Flavobacterium* spp., *Pseudomonas* spp., *Bacillus cereus, B. licheniformis, B. pumilus, B. brevis*, and *B. megaterium*. Of those bacteria, all except *E. aerogenes* are proteolytic when tested on gelatin or peptonized milk (Breed et al 1957).

Baker's yeast, another ingredient of cracker sponges, has its own microflora. According to Peppler (1960), compressed yeast contains 69-71% moisture and a cell count of 20-24 billion cells of yeast per wet gram. He reported that the bacterial content varied but was usually less than 500 million per wet gram of yeast. Cocci that produce lactic acid are always present in either compressed or dry yeast, numbering from 10,000 to several million per gram (Matz 1972).

We wanted to study the interaction of yeast and lactic acid bacteria in dough and flour-water systems. In addition, the effects of proteolytic bacteria and flour enzymes on dough rheology were studied.

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MATERIALS AND METHODS

Yeast

Preparations. The following commercial active dried yeast preparations were used: Fermipan (Gist-Brocades, Delft, Holland), Nugget (Nugget Distribution, Inc., Stockton, CA), Redstar, and Fleischmann's.

Isolation of Pure Culture Yeast. Yeast grew on V-8 agar as raised, white colonies. Single isolated colonies were picked (from Fleischmann and Nugget brands) and maintained on malt agar until used in dough.

V-8 Medium for Lactobacilli. The medium of Fabian et al (1953) consisted of the following: 500 ml of filtered V-8 juice, 10 g of tryptose, 5 g of lactose, 3 g of beef extract, 0.1 g of bromcresol green, and 1 ml of distilled water. The pH was adjusted to 5.7 with HCl before autoclaving at 250°F for 15 min. Lactic acid bacteria produce green to black colonies with yellow halos and develop to a large size in the presence of lactose. Bromcresol green inhibits most of the bacteria that do not form acid (Fabian et al 1953).

Lactic Acid Bacteria

Enrichment and Isolation. To about 5 g of each yeast or flour sample, 100 ml of suspending liquid was added and the mixture incubated for 2-4 days at 30°C. The suspending liquids were: acetate buffer (pH 4.0, 0.2*M*), distilled water with added lactose (1%), or nutrient broth and 1% lactose dissolved in acetate buffer (pH 4.7, 0.2*M*). Because the material was to be viewed with a phase contrast microscope, the suspended material was not shaken and the liquid above the solids was examined to determine the morphology of the growing bacteria (Table I). When enough bacteria were growing, a loopful was streaked on V-8 agar and the plates examined for typical lactic acid bacteria colonies. Cells from typical colonies were also examined under the phase contrast microscope to confirm the type isolated. The best development of lactic colonies was observed after 2-3 days of incubation.

Production. The V-8 broth (without agar) was used to produce lactic acid bacteria. Colonies from V-8 agar medium showing acid production and dark green were selected and grown in Lactobacilli agar of AOAC (1958) stab cultures. Twenty-four hours before inoculating the V-8 broth medium, Lactobacilli broth was poured on top of the Lactobacilli agar stab culture. An inoculating needle was used to remove cells from the stab and place them into the broth above the stab. Those samples were incubated at 30°C for 24 hr. The broth was emptied into 200 ml of V-8 broth and incubated at 30°C for 48 hr. The cells were centrifuged, resuspended in distilled water, and used immediately in dough experiments.

Isolation of Proteolytic Bacteria

About 11 g of flour was added to 99 ml of sterile, distilled water, mixed well, and incubated at 30°C. At various times during a 24-hr period, a loop of the mixture was used to streak skim milk agar plates (reconstituted skim milk, 5% solids, added to a liter of nutrient agar). Proteolytic bacteria produce a clear zone around the colonies. Those colonies were picked and streaked on nutrient agar.

The slants were incubated at 30°C. After incubation, the slants were stored at 4°C until used.

Plate Counts in Flours, Slurries, and Doughs

To determine the counts for the different microbial groups, the following agars were used: total bacteria, nutrient agar; lactics, V-8 agar; proteolytic bacteria, skim milk agar; coliforms, violet red bile agar; and yeast, malt agar.

TABLE I
Isolation of Lactic Acid Bacteria from Yeast by Enrichment Techniques

Method	Yeast	Days Enriched	Microscopic Observations[a]
Acetate buffer	Nugget	3	Short nonmotile rods; cocci; occasional nonmotile slender rods
1% Lactose	Redstar	3	Cocci in chains
	Nugget	3	Short, nonmotile rods; cocci in chains; occasionally long, slender nonmotile rods
Nutrient broth, 1% lactose, acetate buffer	Nugget	2	Long slender rods, nonmotile; cocci; singly and diplococci; cocci in chains
	Redstar	2	Short and long, nonmotile rods; cocci in chains
	Fleischmann	2	Short nonmotile rods; diplococci; cocci in chains

[a]Bacteria confirmed on V-8 agar by acid production and typical green colonies.

TABLE II
Influence of Different Levels of Yeasts[a] on pH of Water-Flour Slurries[b]

Yeast added (g)	Time (hr) 0	18	24
None	5.80	4.3	3.7
5	5.95	4.5	4.23
15	5.83	4.5	4.48

[a]Kansas State University (KSU) data with Fermipan dried yeast, average of two.
[b]50 g of KSU flour in 200 ml of sterile distilled water.

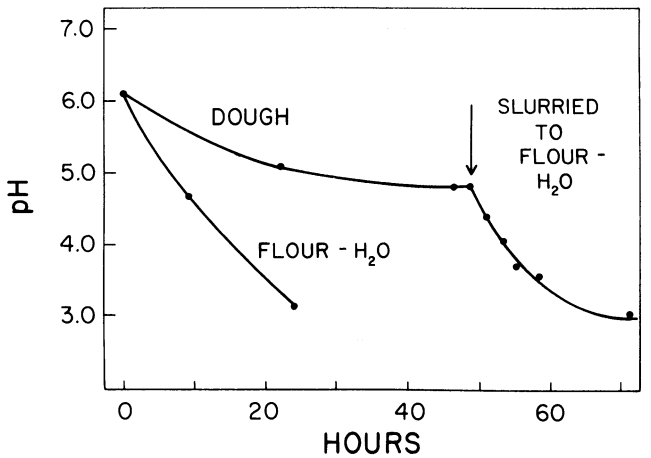

Fig. 1. Decrease in pH as a function of fermentation time for dough, flour-water slurry, and dough slurried to flour-water.

Flour and Dough Experiments

Flours were a commercial all-purpose flour (10.6% protein and 0.56% ash) and a hard red winter wheat flour (9.7% protein and 0.37% ash) milled at the Department of Grain Science and Industry, Kansas State University (KSU). Flour-water slurries were prepared by adding 50 g of flour to 200 ml of sterile, distilled water and incubating at 30°C.

Dough Preparation

Dough was prepared with a KitchenAid model K5-A Food Preparer (Hobart Corp., Troy, OH) equipped with a dough hook. Five hundred grams of flour and optimum water (250 ml for all-purpose or 265 ml for KSU flour) was used to prepare each batch of dough except those containing lactic acid. In those experiments, 25–28 ml of a 1% solution of lactic acid (1 ml of 85% in 100 ml) was added to 50 g of KSU flour, which gave a pH of 3.7. When yeast was used, it was dissolved in some of the added water (usually 50 ml). When pure culture lactic acid bacteria were added to the flour, they were placed in either 250 or 265 ml of distilled water, depending upon the flour used. All doughs were incubated at 30°C, and 100 g of dough was weighed into pint Mason jars and covered.

pH. pH of flour-water mixtures and of blended dough was measured with a Beckman Zeromatic pH meter. One hundred grams of dough and 300 ml of distilled water were suspended in a blender for 1–2 min.

Water Activity. One hundred grams of dough was placed in a pint jar with a gray Hygodynamics sensor connected to an electric Hygometer-Indicator (Hygodynamics, Inc., Silver Springs, MD) to measure the equilibrium relative humidity (ERH). ERH divided by 100 equals water activity.

RESULTS AND DISCUSSION

Yeast Experiments

As a preliminary experiment, different levels of commercial yeast (Fermipan) were added to a flour-water slurry and incubated for 24 hr. The pH of the flour-water slurry without added yeast averaged 3.70, compared with pH 4.23 and 4.48 for the flour-water slurries with 5 or 15 g of added yeast, respectively (Table II). This is more yeast, by weight, than used in cracker dough fermentation, but it showed that the level of yeast influences the pH of flour-water slurries.

Proteolytic bacteria count, as determined with skim milk-nutrient agar, was about the same in Nugget (210/g) and Redstar brands (205/g). However, lactic acid bacteria were found in Nugget brand (2,750/g) and not in Redstar when a flour/water mixture (1:10) was plated on V-8 agar. Both groups—the proteolytic bacteria and the lactics—are found in yeast and are potentially important in bringing about changes in the structure and in the pH of dough.

Plate Counts in Flours

Part of the microflora of two of the flours was estimated by plating slurries on the appropriate agars. The counts for KSU flour were 6,000/g for total bacteria and 100/g for proteolytic. At the 1:10 dilution, no lactic acid bacteria were found but coliforms were at 450/g. Each flour has its own microflora.

Effect of Water on pH

The pHs of flour-water slurries and unyeasted doughs are shown in Fig. 1. Dough pH dropped slowly during 48 hr, whereas pH of a flour-water slurry dropped rapidly. When the dough that had incubated for 48 hr was blended with additional water to give a flour-water slurry of 1:4, the pH also dropped rapidly. Thus, limited water in the dough seems to retard the drop in pH in doughs.

Effect of Yeast and Bacteria

The effects of pure culture yeast (single colony isolate of

commercial yeast) and of lactic acid bacteria on the pH of doughs were studied. Pure culture yeast (5 g, wet basis, per 500 g of flour) had no influence on pH (Fig. 2). The pH of a flour-water slurry decreased more rapidly than did that of dough with or without added yeast. But, when doughs were made with 0.4% commercial yeast and supplemented with high levels of lactics (310×10^6/ml) or when isolated lactics (330×10^6/ml) were added to an unyeasted dough, the pH dropped rapidly. In all cases, the pH plateaued after about 17 hr, which may indicate depletion of fermentables.

Effect of Proteolytic Bacteria

Proteolytic bacteria have been reported in relatively high numbers (10^8) in fermenting cornmeal (Tongnual et al 1981). On the assumption that they also are in wheat flour, we investigated their role in gluten changes during 24-hr fermentations. High numbers of proteolytic bacteria (*Bacillus* sp. isolated from KSU flour) were added to the dough, which was incubated at 30° C for 24 hr. After 24 hr, dough containing proteolytic bacteria could not be removed from the glass jar in one piece because it collapsed and did not retain its shape. The surface of the ball had a glossy appearance. The dough had the consistency of a batter and flowed from a spatula by its own weight. It stuck to the sides of the glass jar and to the spatula and had a sour odor (the mean pH of seven replicates was 5.32). These data show that proteolytic bacteria influence dough rheology when added in high numbers at pH 5.32. However, a test of 25 isolates showed that only 24% (six isolates) grew at a pH of 4.5 and none grew at pH 4.0. Therefore proteolytic bacteria would appear to have only a minimal effect on cracker dough rheology.

Effect of Lactic Acid

Lactic acid was added to dough made with KSU flour to give a pH of 3.7 (well below the pH at which the proteolytic bacteria grow). A control dough was prepared with distilled water. After 24 hr at 30° C, the control dough could be removed from the jar in one piece without breaking. It had a dull appearance and a pH of 5.9. In contrast, the dough containing lactic acid was tacky, had a shiny appearance, and could not be removed from the jar in one piece. Its pH was 3.76. Proteolytic activity of bacteria were prevented at pH 3.7; however, rheological effects similar to those found with proteolytic bacteria were observed. We assume that the rheological changes for the doughs containing lactic acid resulted from native flour proteolytic enzymes. This conclusion is in agreement with that of Pizzinatto and Hoseney (1980).

Microbial Growth in Dough

Dough in which coliforms, proteolytic bacteria, lactic acid bacteria, and the total microflora were measured (Table III) also were analyzed for pH; all had a water activity level of 0.99. Plate count data of flour-water dough (Table III) showed that in the absence of yeast, coliform counts were low, comprising only 0.72% of the total microbial population (estimated by nutrient agar counts). Conversely, 5.4% of the total population consisted of proteolytic bacteria and 27.2% were lactics after 24 hr of incubation.

Introducing yeast changed the microbial relationships (Table III). Bacteria counts were reduced from 18,000,000/g to 100,000/g, and selected bacterial groups such as coliforms, proteolytic bacteria, and lactics were also reduced. Presumably, the competition for carbohydrates or production of inhibiting metabolites by the yeast was responsible for the reductions. Robinson et al (1958) reported two antibiotic substances produced by yeast in preferments.

Yeast counts decreased from 10 million to 570,000/g after 24 hr of incubation. Again the possible explanation is competition for fermentable carbohydrates, toxic effect of low pH, and/or accumulation of toxic waste products.

The data in Table III indicate that the population of lactics is lower in doughs containing yeast (220×10^3) than in flour-water doughs ($4{,}900 \times 10^3$). Populations of lactics in flour-water slurries (Table IV) were much lower ($8{,}900 \times 10^3$) than in similar slurries with added yeast ($105{,}000 \times 10^3$). The phenomena of decreasing yeast counts seen in the dough (Table III) also occurred in the flour-water slurry (Table IV). Those plate count data agree with the pH data (Fig. 2), which showed that the pH of the dough and the

TABLE III
Plate Counts of Different Microbial Groups in Dough at 24 hr With and Without Added Yeast[a]

Treatment	Temperature (° C)	pH	Plate Counts × 10^3/g				
			Yeast	Coliform	Proteolytic Bacteria	Lactics	Total Bacteria
Flour-water	30	5.20	···	130	980	4,900	18,000
Flour-water-yeast[b]	30	5.17	570	0.015	0.33	220	100
Flour-water	4	5.68	···	0.2	0.1	0.3	13
Flour-water-yeast[b]	4	5.46	10,000	0.08	0.08	0.03	<0.1

[a] Dough held at 30° C; plates incubated at 30° C for 48 hr.
[b] Fleischmann's commercial dry yeast.

TABLE IV
Plate Counts of Different Microbial Groups in Flour-Water Slurries[a] With and Without Added Yeast[b]

Treatment	Plate Counts × 10^3/g				
	Coliform	Proteolytic Bacteria	Lactics	Total Bacteria	Yeast
Flour-water					
0 hr	0.09	0.2	0.005	1.2	···
24 hr	23,000	240	8,900	48,000	···
Flour-water-yeast[c]					
0 hr	0.2	0.001	0.001	1.3	31,000
24 hr	16,300	50	105,000	12,900	<100

[a] 200 ml of H_2O in 50 g of flour.
[b] Incubated at 30° C for 24 hr before plating. Plates incubed at 30° C for 48 hr.
[c] Fleischmann's commercial dry yeast.

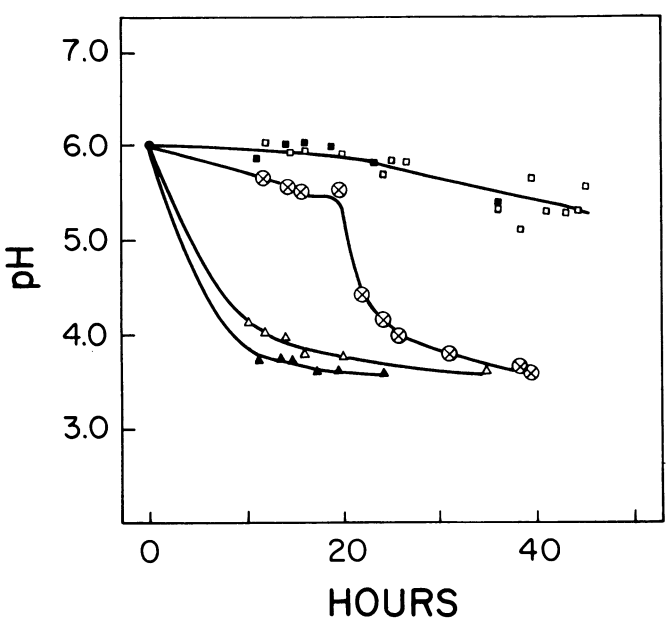

Fig. 2. Effect of fermentation time on pH. ⊗ = flour/water (1:4) slurry; □ = dough containing no added yeast or bacteria; ■ = dough containing pure culture yeast 5 g (wet weight) per 500 g of flour; △ = dough containing added lactic acid bacteria (330×106/ml, 250 ml added); ▲ = dough containing dried yeast (2 g/500 g of flour) plus lactic acid bacteria (310×106/ml, 250 ml added).

dough converted to a flour-water slurry was influenced by microbial physiological activity.

When large populations of lactics were added to the dough, the pH decreased rapidly. Therefore, the use of lactic acid bacteria starter cultures should be desirable in cracker sponge fermentations. Sugihara (1978a, 1978b) showed that frozen concentrates of *Lactobacillus plantarium*, *L. delbrueckii*, and *L. leichmanii* could be used to reduce fermentation time of the sponge from 18 to 4 hr.

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