A NEW METHOD FOR ENUMERATING ROPE SPORES IN BREAD INGREDIENTS

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ABSTRACT

A NEW METHOD FOR ENUMERATING ROPE SPORES

IN BREAD INGREDIENTS

By Gary Alan Houghtby

A new method for determining the number of spores, which cause ropy bread, in flour, vital wheat gluten and other bread ingredients has been proposed. This method is based upon the ability of the vegetative cells to hydrolyze starch by amylases of the cells. With the following medium formulation, a zone of hydrolyzed starch is produced in 24 hours which is visible when the plates are flooded with Lugol's iodine.

Proteose peptone #3	5.0 g
Dextrose	2.0 g
Soluble starch	2.0 g
NaCl	5.0 g
Na_2HPO_4	3.0 g
Agar	15.0 g
Water	1000 ml

pH 7.2 to 7.3

To achieve adequate dispersal of the test materials, especially vital gluten, 0.01 N hydrochloric acid is used as the diluent. The dilution blank, containing the diluent and test material along with a silicone antifoam agent, is heated

at 70 C for 30 minutes to inactivate vegetative cells which would be destroyed during the baking.

The use of this new method makes possible the detection of rope spores from flour, yeast, vital gluten and non-fat dry milk solids which are the major sources. The increase in numbers recovered from vital gluten varied from 2.5 to 2800 times greater than with the accepted test.

From the results of baking tests with ingredients of known spore content, it is estimated that less than 15 spores per gram of dough would result in a satisfactory product. To establish precise standards, more work would be required.

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by

Gary Alan Houghtby

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To Marlene

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INTRODUCTION

For many years ropy bread has, for practical purposes, been unheard of in commercially produced bread primarily because of microbial inhibitors which are added to bread doughs. This does not mean that the baking industry is no longer interested in the number of microorganisms contained in their bread ingredients. Many of the larger companies attempt to secure ingredients of the lowest possible rope spore count as in recent years there have been, on occasion, rope outbreaks in high protein breads. The source of added protein has been vital wheat gluten which also finds use in lower concentrations as a dough strengthener. The bakers felt that the gluten was the source of the rope organisms since they were not having this problem with their other breads.

However, when existing methods for determining the number of rope spores were applied to gluten, consistent results could not be obtained and no correlation of spore counts with bake tests was found. Thus there is need for a dependable method for enumerating rope spores in bread ingredients, with special emphasis placed on vital wheat gluten.

LITERATURE REVIEW

One of the first noticeable characteristics of ropy bread is a strong, somewhat unpleasant odor which has been likened to decomposed melons (Morison and Collatz, 1921). Spoilage begins in the center of the loaf. The spoiled portion becomes soft, sticky, brownish in color and can be drawn out into long threads (Cohn, Wolback, Henderson, and Cathcart, 1919). The spoilage progresses outward from the center of the loaf and if extreme will extend into the crust and soften it. The appearance of odor and stickiness is indicative of the last signs of the spoilage process (Fisher and Halton, 1928).

The odoriferous substances were found by Havanto (1938) to be fatty acids, of which isocaproic was the most volatile. The addition of valine increased the fatty acids yield suggesting that part of the fatty acids is derived from the deamination of amino acids. A second aromatic odor, near the end of the spoilage process, is due to esters formed by reaction of fatty acids with alcohols produced by anaerobic fermentation of glucose in the center of the loaf. Havanto was able to produce these odors <u>in vitro</u> with glucose as the carbon source and ammonia as the nitrogen source.

Fisher and Halton (1928) reported that ropiness is due to the formation of gum and sugar (probably maltose) through the decomposition of starch by amylases. Initial growth of the organisms is dependent upon the presence of soluble nutrients. After these are exhausted the loaf protein is attacked.

Havanto (1940) found that spoilage is accompanied by an increase in water-soluble compounds. The decomposition of starch, first to water-soluble dextrins and later to the reducing sugar maltose, occurs early. He was also able to correlate protein content with decomposition; the higher the protein content, the easier the bread was attacked.

The first extensive investigation of the cause of ropy bread was made by Laurent (1885). He isolated a spore-forming rod from ropy bread and subsequently found it in soil and on cereal grains. The spores of this organism easily survived the temperatures of baking and, when inoculated into bread dough, produced ropy bread. The isolant was found to hydrolyze starch and gluten. Laurent named the organism *Bacillus panificans*.

Kratschmer and Niemilowicz (1889) studied a ropy bread outbreak and isolated *Bacillus mesentericus vulgatus* (Flugge). This organism was found in ropy bread by Uffelman (1890), Russell (1898), Watkins (1906), and Lloyd, Clark, and McCrea (1921). Other organisms of the *Bacillus mesentericus* group which have been isolated include *Bacillus mesentericus fuscus*

(Watkins, 1906), *Bacillus mesentericus niger* (Biel, 1896), and *Bacillus mesentericus ruber* (Kent-Jones, and Amos, 1930).

Other members of the *Bacillus* species which have been isolated include *Bacillus panis* (Fuhrmann, 1906), and *Bacillus liodermos* (Uffelman, 1890).

Vogel (1897) isolated two organisms (*Bacillus mesentericus pani viscocus* I and II. Both caused proteolysis of gluten but only 11 hydrolyzed starch.

Beattie and Lewis (1917) inoculated sterilized bread with pure cultures isolated from ropy bread and were able to produce ropy bread with these isolants. They felt that their cultures were the same species, *Bacillus viscosus panis*, and were able to find it only in flour.

Fifteen aerobic spore-formers which were similar to *Bacillus subtilis* were inoculated into bread doughs by Allen (1919). Rope was produced by all 15 of the organisms in 30 hours when the baked bread was stored at 25 C.

Lloyd, Clark, and McCrea (1921) isolated 5 types of the "mesentericus" group from ropy breads. Starch was hydrolyzed and proteolysis of gluten occurred with each type.

Smith, Gordon, and Clark (1946) made a study of the classification of spore-formers. Cultures of organisms available which had been isolated from ropy or slimy bread were found to be mucoid variants of *Bacillus subtilis*. An extension of this work was reported in 1952 using more sensitive tests for characterization and the classification was not changed.

In the sixth edition of Bergey's Manual (1948), the organisms causing ropy bread were considered to be mucoid variants of Bacillus subtilis as Smith, Gordon and Clark (1946, 1952) had classified them. This classification is continued in the seventh edition of Bergey's Manual (1957).

Several methods have been proposed to determine the presence or the number of rope spores in flour or bread. Almost without exception the tests have been applied to flour only or to the entire loaf of bread with no consideration given to the individual ingredients. Russell (1898) was one of the earliest workers to suspect the presence of rope spores in yeast but was unable to substantiate this.

It remained for Hoffman, Schweitzer, and Dalby (1937) to confirm the presence of rope spores in yeast and other ingredients of bread. The observations were the result of several years' experience in detection and control of rope in a commercial bakery. They found up to 20,000 spores per gram of yeast and were able to correlate the spore content of yeast with ropiness in bread.

Workers in the intervening years concerned themselves with developing new methods for detection of rope organisms and enumeration of rope spores. Watkins (1906) proposed that varying amounts of a suspension of flour in water be added to slices of bread which had been sterilized in tubes. These tubes were then steamed for 30 minutes, incubated at 28 C for 24 hours and observed for rope formation.

Kornauth (1912) baked a loaf from the flour in question and held it at 25 C for 48 hours. If the characteristic odor and stringiness were present, then the flour was considered unsatisfactory. Kornauth also recommended that a known good flour be used as a control.

Voitkevich (1926) reported a method which also used a loaf of bread for the growth medium. For this method, 0.2 g of flour was suspended in 10 ml of water and pasteurized at 75 C for 10 minutes. The contents of the tube were then inoculated into a loaf of bread and incubated for 40 hours at 33 C. Changes in the bread were noted and a microscopic examination made.

Brahm (1921) and Lloyd, Clark and McCrea (1921) proposed the same method. A suspension of 100 g of flour in 300 ml of water was prepared. One ml of the suspension was transfered into a tube of melted nutrient agar and the tube steamed for 20 minutes. The contents of the tube were then poured into a petri dish and incubated at 37 C for 24 hours. Brahm found that less than 12 colonies per ml indicated a satisfactory flour. Lloyd and coworkers found that 12 colonies or less per 4 ml was indicative of a good flour.

Amos and Kent-Jones (1931) reported a variation of the dilution method which used sand to help disperse the flour-in-water suspension and nutrient broth for the growth medium. Dilutions were planted into tubes of broth and the tubes steamed for 30 minutes. Surface growth on the medium at the end of 48 hours was the indicator of the presence of rope organisms. They were unable to correlate the spore content of flour and ropiness in bread and concluded that the processes of fermentation, baking, cooling and storage contributed more to the cause of ropy bread than the organisms present in flour unless the number of organisms was very high.

Hoffman, Schweitzer, and Dalby (1937) published a method which is essentially the same as that of Amos and Kent-Jones (1931). Hoffman _et_ _al_., stated that this method had been privately communicated to Amos and Kent-Jones in 1928 and was the basis for their publication. Hoffman and coworkers were able to establish standards for flour and other ingredients for use in a commercial bakery with their method. They concluded that a count of less than 20 spores per 100 g of flour, less than 100 spores per g of yeast and malt, and less than 10 spores per g of other ingredients allowed production of bread free of rope.

O'Leary and Kralovec (1941) used the Hoffman method for rope spore determinations while studying the effects of calcium acid phosphate and calcium propionate on control of rope in bread. Applying the Hoffman standard to their bread formula, they calculated that 3 spores per g of flour were permissible. The flour used for their bake tests contained no spores and the malt 10 spores per gram as determined by the Hoffman test. Other ingredients were not tested for rope spore content. The table below summarizes their findings. Rope was very slow in developing at 6 spores per gram which was twice Hoffman's standard adjusted to the formula used. The number of spores had to be in the thousands per gram of flour for relatively rapid rope development at the incubation temperature of 32 C.

Table 1. Summary of results of O'Leary and Kralovec (1941)

Spores per gram flour	Days to appearance of: Odor	Color	Viscousness
14000	1.5	3	3
1300	2	3	3
96	3	4	5
55	3.5	4	7*
6	6	7*	8*
0.6	10*	10*	10*

*In isolated spots only.

A method for determining the degree of contamination based on the catalase activity in a sample of bread was proposed by Bunzell and Forbes (1930). The bread was incubated at 50 C for 24 hours or 30 C for 3 days. Twenty-five g of bread and 75 ml of water were mixed and placed in a manometric apparatus. Hydrogen peroxide was added, the pressure changes were measured and the amount of oxygen liberated was calculated. The standard unit was set as the amount of contamination causing liberation in 5 minutes of 1 μg of oxygen from hydrogen peroxide per g of bread.

Bunzell and Kenyon (1932) reported that this catalase method was more sensitive when 6 N hydrogen peroxide was used and the incubation carried out at 40 C. The maximum catalase activity could be achieved in 16 hours incubation.

While testing the effects of several inhibitors on *Bacillus mesentericus*, Wahrmann (1947) was unable to get satisfactory results with the method of Bunzell and Forbes (1930).

Vital wheat gluten, or gum gluten as it is also known, is the protein fraction of wheat flour separated from the starch and other water soluble components by water washing. When dried under low temperature conditions, gluten retains its "gummy" characteristic so that it still has the properties of freshly-washed gluten when it is rehydrated (Carlson and Zeigenfuss, 1958).

Vital gluten is a high protein product, containing up to 80 per cent protein, dry weight, and is used as a protein fortifier. Its primary usage in the baking industry at the present time is as a dough strengthening agent in concentrations of 2 to 3 per cent based on flour weight. It is also used in concentrations of up to 7 per cent in protein breads.

Because of the properties of gluten, particularly its elasticity and cohesiveness, it is almost impossible to make suspensions when water is used as the diluent. Thus, serious difficulties arise when a method used for determining the number of rope spores in gluten requires a water suspension of the test material.

Dilute acids have been shown to be effective dispersal agents for fresh gluten. Wood and Hardy (1908) were among the first workers to examine the effects of acids on gluten. They suspended freshly washed gluten in various concentrations of hydrochloric acid and several weak acids. The rate of dispersion increased up to a maximum as the concentration of acid increased and then decreased until there was no apparent effect. For hydrochloric acid, which was the most effective, they found the 0.03 N solution gave maximum dispersal. Distilled water caused a very slow dispersal which they felt was due to the acidity produced by dissolved carbon dioxide. If salts such as sodium chloride, disodium phosphate and sodium sulfate were added to the acid dispersed gluten, it would then reaggregate with no apparent loss of its original properties.

Upson and Calvin (1915, 1916) also found that strong acids were the most effective dispersal agents and that the maximum adsorption of water occurred with 0.01 N hydrochloric acid. If the temperature of the mixture was increased, the amount of water adsorbed increased. They were able to reverse the adsorption by addition of salts and could prevent the adsorption by addition of a salt before acidification.

It has been recommended by Dill and Alsberg (1924) that a 0.1 per cent sodium phosphate solution, pH 6.8, be used as a wash for gluten as it was the most effective of the salts tested in preventing gluten dispersion.

Detergents which are known to denature most globular proteins have no apparent denaturing effect on gluten protein and do not aid in its dispersal (Blish, 1945).

Dilute hydrochloric acid has been used to aid in dispersal of gluten to free yeast cells for counting while studying the fermentation portion of bread production. Simpson (1936) used 0.05 N hydrochloric acid plus pepsin to suspend gluten and release yeast cells for counting.

Hoffman, Schweitzer, and Dalby (1941) washed the starch from 20 g of dough and added 0.2 ml of concentrated hydrochloric acid to the gluten remaining in 200 ml of water. After standing overnight the gluten was in suspension and the yeast cells released for visual counting.

SECTION I. DEVELOPMENT OF A TEST

Methods for determining the presence of rope spores can be classified into five types which may be either quantitative or qualitative. The first type consists of inoculating a loaf of bread with the organisms which have been isolated from an outbreak of ropy bread or inoculating a dough with the organisms and baking the bread to determine if the spores of the organisms will survive the baking process. The loaf is incubated, cut open and the presence or absence of rope noted.

This method demonstrates the presence of organisms which will cause ropy bread. However, it yields no information about the number of such organisms present in the ingredients of the original loaf nor does it take into consideration the number of organisms necessary to produce a loaf of ropy bread.

The second method consists only of baking a loaf of bread with the suspect flour, incubating the loaf and observing for rope formation. This method also gives no information about the number of spores in the flour or other ingredients of the bread. Thus no decision can be made about the quality of the flour or other ingredients.

The third type of test consists of pasteurizing a dilute flour-water suspension and inoculating a loaf of bread with it. This method does not allow for the presence of spores in the loaf which could have a marked influence on the results, especially if the number of spores were near the critical level for rope formation. It also would result in loss of organisms and flour particles which may contain organisms due to adherence of these to the walls of the tubes. The amount of material adhering to the tube can be quite large due to gelatinization of starch in the flour by the pasteurization process.

The fourth method is based on the catalase activity in a loaf of bread. This method has the same objections as the earlier qualitative methods. The test is applied only to an entire loaf of bread and allows no means of determining the rope spore count on individual ingredients to aid in quality control.

The fifth type of test for rope spores is a dilution method designed for a quantitative determination of numbers. For this test, a dilution of the flour in question is made and portions pipetted into tubes of melted agar. The tubes are steamed to kill vegetative cells and the contents plated. This method has two distinct disadvantages: (1) the adherence of material and organisms to the walls of the tube due to the heating process and (2) retention of some of the agar in the tube. These both result in loss of organisms and material to which organisms may adhere. As the amount of agar remaining varies from tube to tube, this means that a reliable and reproducible count of the spores present in the flour is very difficult to obtain.

A variation of the dilution method which utilizes multiple tube dilutions instead of platings eliminated the two main objections present in the agar method. However, the possibility of entrapment of organisms in the flour particles which stick together when the starch gelatinizes is still present and contributes to the inconsistent results obtained with this method. It also is a relatively non-specific test relying only on heat to eliminate non-spore formers and the ability of the rope organisms to form a pellicle to differentiate it from other spore formers which would survive heat.

The method of Hoffman, Schweitzer, and Dalby (1937), a dilution method utilizing broth, is being used by the milling and baking industries to test their products and ingredients for rope spores. In our hands and in the hands of others this method has not proved satisfactory. Correlation between spore counts of ingredients and ropiness in bread baked with these ingredients could not be obtained.[1]

This has been particularly true when the ingredient in question was vital wheat gluten. Due to the manufacturing

[1]Personal communication from Dr. D. K. DuBois, Manager, Bakery Services, Huron Milling Division, Hercules Powder Co., Harbor Beach, Michigan.

conditions, vital gluten retains most of its properties, particularly stickiness and cohesiveness in water suspension. Thus the purpose of this phase of the study was to find a means of dispersing gluten so that quantitative dilutions could be made of it.

Methodology and Results

The Accepted Method

The method of Hoffman, Schweitzer, and Dalby (1937), hereafter referred to as the Hoffman method, was the comparative standard used through this study. Their test consists of adding 2 g of the material being tested to 96 ml of water and 10 g of sand. This is shaken 50 times and appropriate amounts dispensed into tubes of nutrient broth. The tubes are steamed for 30 minutes, cooled and incubated for 48 hours at 37 C. Surface growth is the criterion of a positive test. The number of rope spores present, per gram, is taken as the reciprocal of the highest dilution with surface growth.

If the number of spores is low enough, larger amounts of material may be tested. This is done by planting directly into 100 ml of broth 1, 2, 5, and 10 g of the material being tested. These samples are steamed for 60 minutes, cooled and incubated at 37 C for 48 hours. After incubation, several loopfuls of each sample are transfered into a tube of broth.

These tubes are steamed for 30 minutes, cooled and incubated at 37 C. Surface growth at the end of 48 hours indicates the presence of rope spores.

The Hoffman method was modified slightly for this study. Five g of the test material and 95 ml of water were used. Dextrose broth (Difco) was selected as the culture medium, rather than nutrient broth, because of its better nutrients. The dilution blanks were shaken 25 times rather than 50 times since the additional 25 times had no effect on dispersal of test materials and 25 times corresponds to standard water and milk testing procedures. When large amounts of material had to be tested, the 1 and 2 g samples were planted in 50 ml of broth and the 5 and 10 g samples in 100 ml of broth. All other parts of the test were performed as previously described.

Isolation of the Test Culture

A culture of organisms causing ropy bread was isolated from a vital gluten sample, lot 818, being tested for rope spores by the Hoffman method. Plates of tryptone glucose extract agar (TGE) (Difco) were streaked from a tube containing surface growth. Individual colonies, which were mucoid in appearance and would form threads when touched with an inoculating needle, were picked into dextrose broth. After 24 hours incubation at 37 C, TGE plates were streaked from these tubes and the 4 cultures which were the most mucoid in appearance were retained for further study.

Smear plates were prepared from 24 hour dextrose broth cultures of the 4 test cultures for spore production. The medium used was TGE with 1 ppm Mn^{++} added to aid in sporulation (Amaha, Ordall, and Touba, 1956). Spores were harvested after 48 hours incubation at 37 C with sterile water, washed 4 times, suspended in 20 ml of water and stored at 4 C.

The spore suspensions were inoculated into 6 rolls each which contained no inhibitors of mold or bacterial growth. The rolls were incubated at 37 C in plastic bags to prevent dehydration. One roll was opened each day and observed for rope formation.

Bread doughs were inoculated with the spore suspensions and the bread baked. The loaves were wrapped in aluminum foil and incubated at 37 C. They were checked daily for the extent of rope formation.

The spore suspension which produced the greatest degradation of the rolls and bread was chosen for further work. A new batch of spores was prepared as above without suspending after the final washing and stored at -18 C until used.

As the initial step in developing a new test, a simple, effective method for suspending vital gluten which would have no deleterious effects on rope spores had to be found. For preliminary studies, solutions of a non-ionic wetting agent Triton X-100, sodium hexametaphosphate, sodium tetraphosphate, sodium tripolyphosphate, hydrochloric acid and acetic acid were used.

The criteria for judging dispersal of gluten were increased volume, decreased particle size, ease of pipetting with a 2 ml pipette, and adherence to the walls of the container as compared to a control using water as the diluent. Three of these four criteria were normally coincident as an increase in volume, representing increased hydration, meant a decrease in particle size and thus easier pipetting.

Five grams of vital gluten was added to 95 ml of the test solution in a dilution blank. The blank was then shaken 25 times and the contents allowed to settle to measure volume changes. The shaking was repeated and portions pipetted observing the particle sizes and also the amount and size of particles adhering to the pipette walls.

Triton X-100 was tested at concentrations of 1:5000 and 1:10,000 and had no effect. The polyphosphates were tested at 0.1 and 1.0 per cent concentrations. Sodium hexametaphosphate and sodium tetraphosphate had no visible effect on dispersal at either concentration. The sodium tripolyphosphate had no effect at 0.1 per cent concentration whereas the 1.0 per cent concentration increased dispersion slightly but adherence of gluten to the walls of the dilution blank and the pipette walls was as great as the control.

The only effective dispersal agents were the acids. They were most effective at these concentrations: hydrochloric, 0.01 N, and acetic, 0.12 N. These resulted in a maximum increase in volume, smallest particle size with resultant ease of pipetting and also there was very little adherence of material to the walls of the vessels. However, if more than a 5 g sample is used, the particle size is not reduced as much and the suspension becomes correspondingly more difficult to work with.

A rather stiff foam, which hindered the mixing action, was produced by the shaking. This foam tended to hold out large particles of gluten, preventing their dispersal. A silicone antifoam agent, Dow Corning Antifoam A, was employed to break the foam. This particular antifoam agent is in a concentrated paste form so that touching the tip of the dilution blank stopper to the agent insured the addition of enough to control the foam. The silicone antifoam agents also aid by decreasing the adherence of materials to containers (Todd, 1956).

Hydrochloric acid, 0.01 N, was used for the following work rather than acetic acid to preclude the possibility of inhibition by the acetate ion.

Using the acid diluent rather than water in the Hoffman method resulted in an increase in counts of at least 100 per cent. However, the gluten, when steamed, formed a mass on the surface of the broth which was difficult to dislodge and interfered with surface growth and observations of surface growth regardless of the diluent used. It also was felt that many organisms would be trapped in this mass and prevented from forming the surface growth indicator. A smaller portion of the gluten adhered to the walls of the tube and also might entrap organisms. When small amounts of gluten were planted in a tube, less than 0.05 g, a thin film of gluten would form on the surface. If this film was not broken up, it was difficult to distinguish from surface growth when the surface growth was very thin and fragile.

During this time a number of other broth media were used. For example, nutrient broth plus 10 per cent sucrose was used in an attempt to utilize slimy or stringy growth as an indication of the presence of rope spores. However, none of these media was any more acceptable than dextrose broth with this method.

With these results in mind, the development of a plating method for determining the number of rope spores present in gluten was undertaken.

Initially attempts were made to utilize the slime production of rope organisms by counting the number of slimy or stringy colonies on a plate. Media which had been used with other organisms to produce polysaccharides (Jeanes, Haynes, and Wilham, 1956; Pederson, and Albury, 1955; Niven, Smiley, and Sherman, 1941) were used without success.

Many of the early workers, who did biochemical tests on their isolants, have shown that the rope causing organisms utilize starch through the presence of amylase enzymes. Smith, Gordon, and Clark (1946, 1952) found that all of the cultures they obtained, which had come from ropy bread, hydrolyzed starch. It was felt that on this basis the hydrolysis of starch would provide a useful indicator for enumerating rope spores.

A dextrose-starch agar was formulated which supported growth of the spore cultures. The amounts of ingredients were adjusted so that a visible zone of starch hydrolysis would be found when the plates were flooded with Lugol's iodine after 24 hours incubation at 37 C.

The medium was then tested with several gluten and flour samples. Most of the organisms present in these samples produced visible zones of starch degradation in 24 hours and there was no indication of interference with the starch-iodine reaction because of the presence of gluten or flour.

As it was often difficult to differentiate colonies from gluten particles, the clearly visible zone of starch hydrolysis, represented by the loss of the blue color of the starch-iodine complex, provided an easily-read test. Other means of differentiating them had been tried in earlier work and were not satisfactory. These included acid-base and oxidation-reduction indicators.

The formulation of the medium as used was:

Proteose peptone #3	5.0 g
Dextrose	2.0 g
Soluble starch	2.0 g
NaCl	5.0 g
Na_2HPO_4	3.0 g
Agar	15.0 g
Water	1000 ml

pH 7.2 to 7.3

A new method of inactivation of vegetative cells was desired because of aggregation of gluten and flour particles when the inoculated medium was steamed. Since heat shocking of spores, as an aid of germination, is carried out at 65 C to 70 C, heating of the dilution blanks containing the test material at 70 C was proposed.

To test this inactivation procedure, a spore-vegetative cell suspension was prepared. The vegetative cells were harvested from a 24-hour culture, starting with a spore inoculum. Seven equal dilutions were prepared in 0.01 N hydrochloric acid. The dilutions were heated for varying times at 70 C and appropriate dilutions planted using 5 plates per dilution. Dextrose starch agar was used to allow determination of the number of amylase positive cells.

About 10 minutes were required for the contents of a dilution blank to reach the desired temperature. After an additional 10 minutes, or a total of 20 minutes, the number of amylase positive organisms present in the suspension had dropped to a constant number indicating that all of the vegetative cells had been inactivated (Table 2).

Table 2. Thermal inactivation at 70 C of the spore-vegetative cell suspension

Total time	Number surviving per ml
0 minutes	560
5 minutes	540
10 minutes	130
15 minutes	104
20 minutes	10
25 minutes	11
30 minutes	14

The new method now consisted of the following:

1. Diluent used throughout is 0.01 N hydrochloric acid.

2. Up to a 5 g sample of gluten may be used in a total volume of 100 ml.

3. An antifoam agent is used to reduce foaming which occurs when the suspension is shaken 25 times.

4. Heat the suspensions prepared at 70 C for 30 minutes with shaking at intervals to aid in dispersal.

5. Plate appropriate dilutions with starch agar and incubate for 24 hours at 37 C.

6. Flood plates with Lugol's iodine and count the colonies which have zones of starch hydrolysis surrounding them.

Comparison of Hoffman Method with New Method

The Hoffman method and the new method were first compared using spore suspensions. For this comparison, 1 ml portions of a spore suspension were placed in each of 5 dilution blanks for each method. Five tubes or plates per dilution were used for each determination. Results of two comparisons using the same suspension are presented in Table 3.

Set I was a comparison with the spore suspension only. In set II 5 g of flour was added to each of the dilution blanks along with the spore suspension. The flour was added primarily to check the effect of the heating step of the new method on the starch in flour as heat will cause gelatinization of starch.

There was a partial gelatinization occurring as the viscosity of the suspension increased markedly. However, there was no difficulty in pipetting the suspension as it could be well mixed by shaking. Some agitation during the heating period also aided in diminishing the effects of gelatinization.

The increase in viscosity had no effect on the recovery of spores as the mean of set II was not significantly different from the mean of set I at the 5 per cent level as calculated by Student's T test described by Dixon and Massey (1957).

The more important result was the much greater recovery of spores with the new method than with the Hoffman method. This occurred both in the absence and presence of flour.

Table 3. Comparison of Hoffman method with new method using a spore suspension and flour

Set	Sample	Hoffman method[1]	New method[2]
I	A	1×10^6	27.2×10^6
	B	1×10^5	36.2×10^6
	C	1×10^5	30.0×10^6
	D	1×10^5	26.6×10^6
	E	1×10^5	34.0×10^6
	Mean	2.8×10^5	30.7×10^6
II[3]	A	1×10^5	41.2×10^6
	B	1×10^5	26.6×10^6
	C	1×10^5	40.8×10^6
	D	1×10^5	37.0×10^6
	E	1×10^5	34.8×10^6
	Mean	1×10^5	38.1×10^6

[1]Reciprocal of highest positive dilution.

[2]Plate count.

[3]Five g flour added.

This type of comparison was repeated using spores, spores plus 5 g of flour, and spores plus 5 g of gluten. Three plates or tubes per dilution were planted. The results of 2 sets of tests are presented in Table 4. Again there was approximately a 100 times greater recovery of spores by the new method over the Hoffman method. The means of the three determinations by each method within a set are not significantly different at the 5 per cent level as calculated by the analysis of variance test described by Dixon and Massey (1957).

The two methods were compared on 25 gluten samples. Again 3 plates or tubes per dilution were planted and the counts of duplicate sets averaged. The results are presented in Table 5.

It can be seen that in general there is a rather large difference between the counts obtained by the two methods on each gluten sample. In all cases the new method gave the larger number which was 2.5 to 2800 times greater than the number using the Hoffman method.

It is also evident that there is no correlation between the two methods. As an illustration, the Hoffman method count for lot 2176 was 1000 spores per 100 g whereas the new method count was 6000 spores per 100 g. Lot 2363 also contained 6000 spores per 100 g by the new method but only 10 spores per 100 g by the Hoffman method.

Table 4. Comparison of Hoffman method with new method using spore suspensions and flour or gluten

Set	Sample	Spores		Spores plus flour		Spores plus gluten	
		Hoffman method	New method	Hoffman method	New method	Hoffman method	New method
		spores $\times 10^{-5}$	spores $\times 10^{-6}$	spores $\times 10^{-5}$	spores $\times 10^{-6}$	spores $\times 10^{-5}$	spores $\times 10^{-6}$
I	A	1.0	29.3	1.0	25.0	1.0	26.3
	B	1.0	28.7	1.0	22.7	1.0	23.0
	C	1.0	25.7	10.0	31.3	1.0	31.3
	D	1.0	23.7	1.0	32.0	10.0	33.7
	E	1.0	36.6	1.0	27.3	1.0	34.0
	Mean	1.0	28.8	2.8	27.7	2.8	29.7
II	A	10.0	99.3	10.0	89.7	1.0	136.7
	B	1.0	91.0	10.0	127.3	1.0	116.7
	C	10.0	91.7	10.0	90.0	0.1	79.7
	D	10.0	77.3	10.0	116.7	10.0	116.3
	Mean	7.75	89.9	10.0	105.9	3.0	112.4

Discussion

The method of Hoffman, Schweitzer, and Dalby (1937), which is accepted by the baking and milling industries, has been found inadequate for determining the number of rope spores in gluten. Modifying this procedure by using 0.01 N hydrochloric acid as the diluent to disperse adequately the gluten still did not provide a satisfactory test even though recovery of spores was increased. The gluten came to the surface of the broth when steamed and formed a solid mass on the surface which was very difficult to dislodge and interferred with pellicle formation, the sole criterion of rope spore presence.

A new plating procedure was developed using a dextrose-starch agar for the medium. This enabled utilization of starch hydrolysis, by amylases produced by the organisms, as the indicator of rope spore presence. By flooding the plates with Lugol's iodine after 24 hours incubation at 37 C, the number of clear zones, representing areas of starch hydrolysis by individual colonies, could be counted and the number of rope spores present in the sample calculated.

Inactivation of vegetative cells in the test material, simulating inactivation by baking, was done by heating a dilution blank containing 95 ml of 0.01 N hydrochloric acid and 5 g sample of the test material at 70 C for 30 minutes.

Table 5. Comparison of Hoffman method with new method on 25 gluten samples

Gluten lot	Hoffman method spores/100 g	New method spores/100 g	Ratio: New method / Hoffman method
2176	1000	6000	6.0
2179	2000	5000	2.5
2186	20	4000	200.0
2187	10	15500	1550.0
2199	50	15000	300.0
2245	50	12000	240.0
2252	1200	16000	13.3
2269	550	32000	58.2
2272	250	25000	100.0
2278	225	4000	17.8
2281	100	10000	100.0
2287	75	10000	133.3
2293	55	10000	1818.2
2302	25	4000	160.0
2305	50	12000	240.0
2307	400	3000	7.5
2311	60	2000	33.3
2315	50	20000	400.0
2320	210	2000	9.5
2326	250	4000	16.0
2338	10	28000	2800.0
2344	15	10000	666.6
2353	205	1000	4.9
2359	250	5000	20.0
2363	10	6000	600.0

The new method was compared with the Hoffman method using spore suspensions in the presence of flour or gluten. Up to 100 times more spores were found by the new method. When the two methods were compared using 25 gluten samples, the new method gave the larger number in all cases which was from 2.5 to 2800 times greater than that obtained by the Hoffman method.

On the basis of these results, the new method is a better procedure for determining the number of rope spores present in bread ingredients. The method of dispersal of the test materials should allow a more accurate determination of spore numbers permitting standards to be set on bread ingredients.

SECTION II. APPLICATION OF THE NEW TEST TO A STUDY OF ROPE FORMATION IN BREAD

Once a better method for determining the rope spore content of bread ingredients has been found, it becomes necessary to apply this method with baking tests to establish standards for the rope spore content of bread ingredients. By using ingredients of known spore content and augmenting with graded doses of a spore culture, it should be possible to determine the number of spores required to cause the development in a loaf of bread of a definite level of spoilage in a given time period. The results of such a study are presented in this section.

Materials and Methods

The following bread formulation was used throughout the work.

Yeast	7.5 g
Non-fat dry milk solids	23.0 g
Sugar	14.0 g
Fat (Crisco)	24.0 g
Salt	7.0 g
Flour	460.0 g
Flour for kneading	10.0 g
Water	300.0 g

The procedure for making the bread was as follows:

1. The yeast was sprinkled over the water, at 30 C, and allowed to stand for 5 minutes.

2. The dry milk solids were added to the yeast mixture and beaten 20 seconds with a Kitchen Aide model K5A mixer at setting 1 using the wire whip attachment. All subsequent mixing was done with the mixer at setting 1.

3. The salt, sugar, and fat were added and beaten for 30 seconds.

4. The flour was added in 3 portions:

    a. 230 g, beat 2 minutes, scrape bowl.

    b. 115 g, beat 2 minutes, scrape bowl.

    c. 115 g, beat 1 minute, scrape bowl.

    d. Beat 1 minute.

5. The dough rested in the bowl for 5 minutes before being turned out onto a lightly floured surface and kneaded for 5 minutes.

6. The kneaded dough was placed in a greased bowl, covered and proofed for 60 minutes at 35 C.

7. The dough was then turned out from the bowl, punched down, kneaded for 1 minute and returned to the bowl for proofing for 30 minutes.

8. The dough was again turned out, punched down and kneaded 25 times before dividing into three equal portions.

9. The three portions were shaped into loaves, placed

into pans and proofed for 30 minutes before baking at 400 F for 45 minutes.

10. After baking, the loaves were turned out of the pans, allowed to cool for 20 to 30 minutes and wrapped in aluminum foil for incubating at 35 C.

The loaves were examined every 24 to 48 hours for rope. At each examination one of the three loaves per batch was cut or broken open and the degree of rope infection judged according to the following scale.

(0) No apparent change in odor, color or constitution.

(1) Slight odor with a few isolated, very small brown spots.

(2) Many large brown spots with decided odor. Spots are mucoid and will string.

(3) Center of loaf discolored, very mucoid and sticky.

(4) Entire loaf including crust is involved.

At the (1) level, the consumer probably would not notice the odor as it is slight. Nor would the brown spots probably be noticed as they are very few in number and very small. This was the normal condition of the control loaves, when the methodology had become standardized, after 6 to 8 days incubation. The odor and discoloration in spots became readily noticeable at the (2) level and stickiness may be apparent in spots. At the (3) level, the interior of the loaf has become degraded so that it is extremely difficult to cut. When the loaf is broken open, the center forms "strings" or "threads" which may be drawn out to 12 inches or more before breaking. The characteristic odor, somewhat like spoiled fruit, is very strong.

When the (4) level is reached, the loaf shape has broken down and there is a mass of sticky, mucoid, discolored material remaining.

The appearance of the (3) level of rope within 3 days was the desired response in these bake tests. The characteristic odor was present long before any easily visible signs such as discoloration appeared. However, odor as such, was not considered a reliable sign due to the proximity of loaves which were in advanced stages of decay and giving off strong odor which could be adsorbed by other loaves. Frequently, a stickiness of the crumb was noticeable with the faint early odor. This stickiness may have been due to the breakdown of starch to dextrins and other water soluble products. It usually was found when the (3) level of rope was to be found in 3 to 4 days.

Initially spore counts were done of the flour and yeast which had 4 per gram and 35 per gram respectively. This meant that the flour used contained, per batch, about 1900 spores and the yeast about 300 spores. The new method only was used for spore counts in this study.

While developing the ability to make reproducible loaves, several loaves were incubated for 6 days and were found to contain spots of rope corresponding to the (2) level. As it was desired to be able to hold bread this long, the smaller the amount of residual activity in the ingredients, the better the test would be. Reports in the literature, especially O'Leary and Kralovec (1941), indicated that a much larger number of spores than the approximately 2200 contained in the flour and yeast were necessary to produce the degree of rope found.

Spore counts were made on the other ingredients and the only one with an appreciable number was the milk solids, which contained 400 spores per gram. Thus, more than four times as many spores were being added in the milk as in all of the other ingredients combined.

As these milk solids had been obtained from the Michigan State University Dairy, other dried milk samples were purchased in local stores and found to contain comparable spore counts.

Thereafter, the milk solids were reconstituted in 150 g of water and sterilized in the autoclave at 116 C for 15 minutes. This produced minimal browning of the milk and had no apparent effect on the bread quality.

Bread made with the sterilized, reconstituted milk showed only a very few isolated brown spots corresponding to the (1) level of rope infection after 6 to 8 days incubation at 35 C (Table 6). There was no visible change in the control loaves after 3 days incubation when the loaves inoculated with spores would show heavy rope formation or the (2) to (3) level.

The usual procedure followed was to prepare two batches, each of which contained 3 loaves, of bread. One batch would be inoculated with spores, which were added at the same time with the dried milk. The second batch usually was not inoculated and served as the control. However, during the early portions of the bake tests, each batch was inoculated with a different level of organisms to establish a general range of spore activity.

To eliminate the possibility of passing spores from the first batch to the second, 2 sets of bowls, utensils, trays, and pans were used; one for each of the batches. As the two batches were normally prepared concurrently, rubber gloves were worn when handling the doughs to prevent cross contamination. One pair of gloves was used with each dough and the gloves changed each time a different dough was handled.

The kneading and shaping of loaves were done on trays so that the doughs never came into direct contact with any surfaces in the laboratory which could not be autoclaved. All bowls, utensils, pans and other materials were sterilized by autoclaving after use.

Two spore preparations were used in the bake tests. The first, designated 11/30, was used for the earlier developmental work and throughout the baking. The second, designated 4/26, was prepared after the bake tests had been started. The first preparation was used as the inoculum for the second. The 4/26 spores were prepared and stored as previously described.

Results

With spore preparation 4/26 it is evident that increasing the number of added organisms from 1800 to 36,000 materially increased the rate and amount of rope formation (Table 7). This does not seem to occur with preparation 11/30 as an increase from 80 to 800 or even 80,000 added spores shortened the time needed to reach a given level by only 1 day and this was the (3) level which equivalent to badly decomposed bread.

The difference in activity between cultured spores and those naturally present in bread ingredients is very great. The addition of 80 of the 11/30 spores to a dough producing spoilage at the (3) level in three days opposed to the 9200 spores present in the milk used requiring six days to reach the next lower level of spoilage is an example of this difference.

Table 6. Results of bake tests

Initial spores	Spores added		Total spores	Spores/g dough	Level of rope					
	Gluten	Milk			3 days	4 days	5 days	6 days	7 days	8 days
2200[1]			2200	2.6	0			1		1
2200	3000		5200	6.0			1		2	
2200		9200	11400	13.5		1		2		

[1] Spores in flour and yeast.

Table 7. Results of bake tests with spore suspensions

	Spores added				Level of rope				
Initial spores	Spore suspension	Gluten	Total spores	Spores/g dough	1 day	2 days	3 days	4 days	6 days
2200[1]	80[2]		2280	2.7	1		3		
2200	800		3000	3.5	1		3		
2200	8000		10200	12.1	2	3			
2200	80000		82200	97.2	2	4			
2200	800	3000	6000	6.9	1	3			
2200	800[3]		3000	3.5			0	2	
2200	3600		5800	6.9			1	2	
2200	36000		38200	45.2	1	3		4	
2200	180000		182200	215.5		3	4		
2200	360	3000	5560	6.4				1	3

[1] Spores in flour and yeast.

[2] 11/30 spore preparation.

[3] 4/26 spore preparation.

The 4/26 spores were also more active in producing spoilage than the naturally occurring spores although the response was slower than that of the 11/30 spores. The addition of 3600 of the 4/26 spores resulted in a (2) level of rope in 4 days whereas the milk spores needed six days to reach this level of spoilage and were 2.5 times greater in number.

Gluten was added at a concentration of 4.25 per cent, based on flour weight, to a few batches to see if any effect due to the spores it contained could be detected. Twenty grams of lot 2199, which contained 150 spores per g, was used. The 3000 spores added had a small but definite effect on rope formation. The degree of spoilage increased from (1) in 8 days for the control to (2) in 7 days for the gluten loaves (Table 6).

The addition of a few organisms to dough with added gluten increased the rapidity of rope formation. The addition of 360 spores of the 4/26 preparation, resulting in a total of 5560 spores, produced a (3) level of spoilage in 6 days compared to the (2) level in 7 days for the gluten loaves. This (3) level in 6 days is comparable to the (2) level in 4 days which 3600 of the 4/26 spores reached.

Discussion

The data presented in Tables 6 and 7 illustrate the effect of increasing spore concentration on rope formation. The increase from 2.6 to 6.0 to 13.5 spores per g of dough resulted in a shortening by one day, for each increment, to the first signs of rope formation. This indicated that less than 15 spores per g of dough should result in production of bread which would remain rope free for the time normally required for consumption.

The difference in response of the two spore preparations is difficult to explain. There is undoubtedly a natural variation in activity of the spores present in different gluten samples. In choosing the initial spore culture, the one with the most mucoid appearance and best rope producing ability was chosen. This may have created unwanted bias toward finding an artificially low number of spores necessary for rapid rope formation.

The difference in response of the two spore preparations indicates that the ability to degrade protein and produce rope can be modified on subculture. The indications are that the 4/26 preparation more nearly gives the true picture with regard to the number of spores required for rapid rope formation than the 11/30 preparation.

However, the bake test results indicate that the number of 4/26 spores required for rope formation is lower than would be expected on the basis of results presented in Table 6 with naturally occurring spores. It is apparent that the initial culture chosen for this study is not typical of the normal spore population of bread ingredients. On subculture, it became more typical of the normal population.

This makes establishment of a definite number of spores necessary to cause the development of a given level of rope in a given time difficult. It appears that less than 15 spores per gram of dough should cause no difficulty. More work would be required to establish precise standards.

In future work for setting standards, the difference in temperature between that used in these studies and that of normal bread storage should be considered. While doing the preliminary baking studies when choosing a culture to work with, some of the loaves were held at room temperature, 23 C to 25 C. These loaves were inoculated with very active cultures in large numbers and they required at least 1 additional day to reach the same level of rope formation as those loaves incubated at 37 C.

It also must be borne in mind that the estimate is based upon the bread formulation used for this study. This estimate would have to be adjusted if another formulation were to be used.

SUMMARY

A new method for determining the number of spores in flour, vital wheat gluten and other bread ingredients which cause ropy bread has been proposed. This method is based upon the ability of vegetative cells to hydrolyze starch due to the presence of amylases. With the dextrose-starch agar medium used, a zone of starch hydrolysis is produced in 24 hours which is visible when the plates are flooded with Lugol's iodine.

To achieve adequate dispersal of the test materials, especially vital gluten, 0.01 N hydrochloric acid is used as the diluent. The dilution blank, containing the diluent and test material along with a silicone antifoam agent, is heated at 70 C for 30 minutes to inactivate vegetative cells which would be destroyed during baking.

This new method results in increased numbers of rope spores being recovered from flour, yeast, vital gluten and non-fat dry milk solids which are the major sources. The increase in numbers recovered from vital gluten varied from 2.5 to 2800 times greater than with the accepted test.

From the results of baking tests with ingredients of known spore content, it is estimated that less than 15 spores

per gram of dough would result in a satisfactory product. To establish precise standards, more work would be required.

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