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To the Graduate Council:

I am submitting herewith a thesis written by Kadumpalli Anjan Reddy entitled "The Use of the Oxidase Test for Enumerating Psychrophilic Bacteria in Cottage Cheese." I recommend that it be accepted for nine quarter hours of credit in partial fulfillment of the requirements for the degree of Master of Science, with a major in Food Technology.

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THE USE OF THE OXIDASE TEST FOR ENUMERATING PSYCHROPHILIC BACTERIA IN COTTAGE CHEESE

A Thesis

Presented to

the Graduate Council of

The University of Tennessee

In Partial Fulfillment

of the Requirements for the Degree

Master of Science

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by

Kadumpalli Anjan Reddy

June 1973

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ABSTRACT

The 75 samples of Cottage cheese secured from manufacturers and retail outlets in Tennessee over a period of six months were analyzed to determine whether the oxidase count of the Standard Plate Count was a true reflection of the Psychrophilic Plate Count obtained by the standard procedure. The purpose of this investigation was also aimed to consider the feasibility of the oxidase test as a substitute to standard procedure for enumerating psychrophilic bacteria.

Analysis of the data with paired t test indicated the presence of a highly significant difference between the oxidase test and the standard procedure. Observations revealed that oxidase positive organisms constituted only 38 percent of the Standard Plate Count and 17 percent of the Psychrophilic Plate Count. Some of the psychrophilic plates containing more than one million psychrophiles per gram of cheese were negative for oxidase test.

The study gave an impression that oxidase test was not giving a true representation of psychrophilic microorganisms involved in the spoilage of Cottage cheese. This investigation also raised the question whether all Pseudomonas organisms involved in the spoilage of Cottage cheese are capable of showing the oxidase positive characteristic.

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CHAPTER I

INTRODUCTION

The biochemical characteristics of microorganisms play an important role in assessing the quality of a food product. Identification of oxidase reaction in certain microorganisms can be regarded as an important biochemical supplement to the already existing characteristics such as proteolytic, lypolytic, saccharolytic, acid and gas producers. These biochemical activities of microorganisms become an interesting topic for food microbiologists and equally a topic of concern to the food manufacturing entrepreneur in view of product deterioration. Each biochemical activity of microorganisms signify a particular characteristic and the oxidase reaction may be classified among them.

A well recognized fact has been generally accepted that psychrophilic microorganisms play an important role in storage life and in keeping quality of refrigerated food products. These microorganisms are responsible for extensive changes in the physical and organoleptic characteristics of food resulting in unclean, winey, fruity, rancid, and putrid flavors (23,30,45,55). The subject of enumeration of psychrophilic microorganisms is still a matter open for debate. Several research workers (10,13,55) have proposed their own definition and method of enumeration, indicating a lack of agreement on this subject. Hence, Baumann and Reinbold (2) reported that no single definition has been universally accepted for these organisms. Standard Methods for the Examination of Dairy Products (1) for psychrophilic bacterial count,

recommends holding plates at 7 C for ten days. This long incubation period has been of great concern to the dairy industry, for any attempt to identify the contaminant after such a long time is not only difficult, but of little value. In recent years, research workers have attempted to determine the potential psychrophilic count within 48 hours based upon the oxidase positive characteristic of microorganisms (24,25,26,27). This oxidase reaction has been attributed to the presence of a cytochrome c oxidase enzyme system in bacteria (32). In view of the oxidase positive nature of Pseudomonas organisms (8,24,53), which comprise the largest group of psychrophiles (30,55), the oxidase test has been reported to be an effective means for discovering the potential psychrophilic problem in refrigerated food products.

The present study was conducted to determine the feasibility of the oxidase test as a substitute for the standard procedure for psychrophiles, if the oxidase count of the microorganisms involved in the spoilage of Cottage cheese gives a reliable reflection of the psychrophilic count obtained from the standard test. Research data of this type may provide evidence of the extent of reliability on the oxidase test in determining the psychrophilic count of refrigerated food products.

CHAPTER II

REVIEW OF LITERATURE

A cursory examination of the literature is in conformity with the views expressed by Castell and Garrard (8) that relatively little attention has been paid to the "oxidizing capacity" of microorganisms in relation to the spoilage of dairy and other food products. Early studies revealed the potential usefulness of the oxidase reaction in the identification (20), taxonomical classification (53) and differential diagnosis of (19) microorganisms. In recent years, attempts have been made by research workers (24,25,26) to point out the potential usefulness of the oxidase test in the food industry.

I. HISTORICAL

The phenomena of oxidation and reduction in bacteria were observed in the early 1900's. Early contributions to this study were made by several research workers (12,14,17,22,48) who emphasized the importance of oxidase reaction in bacteriology. Further emphasis on this type of study was pursued by Keilen (32), who noted the presence of a cytochrome oxidase-cytochrome c system responsible for oxidation and reduction in bacteria, and further reported that the extracts of heart muscle, yeast cells, and certain bacteria are the potential sources for cytochrome pigments, a finding which was later confirmed by Smith (49), and Smith and Stotz (51). Later, increasing interest in the study of the oxidizing

capacity of microorganisms was attributed to the investigations of several research workers (8,20,24,25,26,34).

II. BACTERIAL CYTOCHROMES

Cytochrome is an intracellular respiratory catalyst common to animals, bacteria, yeast and higher plants (32). Keilin (32) proved that these pigments are not simple compounds, but a complex formed of three distinct haemochromogen compounds. Therefore, he proposed the name of cytochrome signifying merely "cellular pigment." Smith's (49,50) spectroscopic observations revealed that these pigments may be characterized by sharp bands in the adsorption spectrum if they were in reduced form, and that the appearance or disappearance of these bands could be seen with a visual spectroscope. The position of adsorption spectrum has been shown to be the same in all organisms, but the highest concentration of pigment has been found in the thoracic wing muscles of flying insects, the striated muscles of mammals and birds and in baker's yeast. These cytochrome pigments have been classified as a, b, c, and d, but observations however, have revealed that these pigments are subdivided and that some of them exist in combination.

Keilin and Hartsee (33,34) demonstrated that cytochrome c oxidase enzyme of the heart muscle catalyzes the oxidation of cytochrom c by oxygen. Furthermore, the cytochrome oxidase-cytochrome c system was found to oxidize a number of substances such as the "Nadi" reagent (dimethyl p-phenylenediamine plus alpha naphthol), p-phenylenediamine and hydroquinone. Smith (49), while observing the same type of reaction

in certain bacteria containing the cytochrome oxidase-cytochrome c system, cautioned that the oxidation of these sybstances cannot be taken as sole evidence for the presence of cytochrome c oxidase.

The cytochrome oxidase enzyme has been known under several names, such as atmungsferment, indophenol oxidase, nadi oxidase, cytochrome a_3 oxidase, cytochrome a oxidase, and cytochrome c oxidase (20). The enzyme has been shown to be associated with insoluble materials within the cell and has resisted purification (49). Subsequent studies by Smith and Stotz (51) however, have indicated that the enzyme could be extracted by employing a cholate extract with digestive enzymes, under carefully controlled conditions. This procedure has led to an increased solubility of the enzyme, so that fractionation by ammonium sulfate precipitation could be easily achieved.

The presence of cytochrome pigments in microorganisms does not necessarily reflect the presence of oxidase enzyme; thus many of the bacteria which contain cytochrome pigments are negative for the oxidase enzyme. Smith (50) reported no significant cytochrome c oxidase activity in the members of Acetobacter, Aerobacter, Bacillus, Escherichia, Sarcinia, and Micrococcus, whereas, the suspensions of broken yeast cells and heart muscle particles showed greater activity. Hence, the respiratory enzymes of the bacteria were found to be different from those of yeast and mammalian tissues. When the respiration of bacteria was expressed in terms of the cytochromes present, some of the bacterial systems were found to be considerably more active (have higher turnover numbers) than those of yeast or the heart muscle. Vernon (54) isolated

cytochrome pigments from the extracts of Rhodospirillum rubrum, while Lenhoff and Kaplan (38) isolated cytochrome pigments from the extracts of Pseudomonas fluorescens.

Oxidase activity of bacteria has been proven to be inhibited by cyanide, azide, sulfide, and carbon monoxide compounds, but the competitive inhibition of respiratory enzymes by carbon monoxide has been reversed by light (49).

III. OXIDASE REACTION

Keilin and Hartsee (34) showed that the addition of cytochrome c to a heart muscle preparation catalyzes the oxidation of substances such as p-phenylenediamine, hydroquinone, cysteine, and ascorbic acid. The oxidation of these substances were shown to be catalyzed not by the oxidase alone, but through the cooperation of cytochrome c. Hence, the name "cytochrome oxidase" was proposed.

Different types of paradiamines have been used since the early 1900's for studying the oxidation and reduction in bacteria. Available evidence indicates that as early as 1902, Dietrich and Liebermeister (12) observed the production of indophenol by the oxidation of dimethyl p-phenylenediamine hydrochloride and alpha naphthol upon applying this reaction to demonstrate the details of structure in the anthrax bacillus. An interesting study by Dunn (14) on the oxidase reaction in myeloid tissues revealed that the reaction was of great value in demonstrating the distribution of myeloid tissue in normal foetal organs and of granular leucocytes in the organs in myeloid leukemia. Schultze (48)

and Kramer (36) applied the oxidase reaction for observing the oxidation and reduction in bacteria, and Callow (6) attributed the oxidase reaction to the presence of a heat-stable peroxidase substance in certain bacteria further concluding that his findings were similar to those of Keilin (32) in his work on cytochromes.

Gordon and McLeod (22), in their investigation regarding the practical application of the direct oxidase reaction in bacteriology, concluded that the reaction was a good device for rapid differentiation of certain bacteria in mixed cultures. Smear preparations from colored colonies indicated no change in staining characteristics, suggesting that the reaction was possibly at the surface of the bacterial cell. The isolation of bacteria was a difficult problem in view of the antiseptic nature of the dye to bacteria, and suggestions were made to prepare a dye with less toxicity, yet having the capacity to yield color pigments on oxidation.

In a later study on bacterial oxidations, Ellingworth et al. (17) attempted to diminish the toxicity of diamines by sulphonation and also to increase the sensitiveness of the reaction by combining the diamines with aniline, or its ortho-sulphonic acid. Though the toxicity was found to be diminished, a simultaneous loss of sensitiveness to bacterial oxidation occurred. Bacterial action of aniline diamine mixtures gave negative results when incorporated in the medium even though they are more sensitive reagents. The speed with which the death of bacteria occur has a close relationship to the rapidity of color development in the colonies. The interval between exposure and death of bacteria has

been reported to range from 2 to 30 minutes, depending upon the nature of dye, and tetramethyl p-phenylenediamine hydrochloride was reported to be the least toxic. Unless the reagent was completely protected from air and light, spontaneous oxidation resulted forming a mixture of substances of varying bactericidal power.

Early studies on the oxidase reaction indicated that the reaction depends on the presence of both peroxide and peroxidase in the bacterial cell. Callow (6) described a heat stable peroxidase of bacteria capable of producing the color reactions. The reaction was more common among the bacteria which grew well in the presence of oxygen containing a thermolabile direct oxidase system. Dunn (14), demonstrating the oxidase reaction in myeloid tissue, described the investigation of Brandenberg (5), who opined that the oxidizing substance in leucocytes was in the nature of a ferment and that it had the constitution of a nucleo-albumin. Carpenter et al. (7) reported that the oxidase reaction depends upon the reaction of an oxidative enzyme with an aromatic amine to produce a series of readily distinguishable color changes ranging from pink to black. The oxalic acid salts of aromatic amines were, in general, more stable than the corresponding hydrochloride acid salts and hence the oxalate salt of p-aminodimethylaniline was recommended as an improved reagent in the oxidase reaction.

The concept of mechanism of oxidase reaction has taken a new direction with the findings of Keilin (32), who reported that the reaction specifically resulted from the presence of cytochrome oxidase-cytochrome c system in bacteria.

Steel (53) reported that the oxidizing capacity of any living specimen plays an important role upon the presence of cytochromes, which are a "group of respiratory pigments comprising iron-porphyrin compounds." Most aerobic and facultatively anaerobic bacteria contain this cytochrome system which acts as an electron carrier in aerobic respiration. Furthermore, Steel (53) concluded that distribution of these cytochromes vary with different bacteria; some have several, while others have only one or none at all.

IV. BACTERIAL OXIDATIONS OF PARADIAMINES

Paradiamines yield a variety of colored oxidative products the precise nature of which depends upon the oxidizing agent used and the condition of reaction. Colored substances such as Wurster's red and Wurster's blue have been described by Ellingworth et al. (17) who reported increased oxidation for dye containing an increase of methyl groups.

Castell and Garrard (8) observed that p-phenylenediamine hydrochloride changes to a colored form at low Eh and, therefore, described it as more sensitive and more quickly acting. Weak oxidizing organisms gave positive results with the tetramethyl compound, whereas, negative results have been noticed when p-aminodimethylaniline hydrochloride was used.

Gaby and Hadley (20) developed a laboratory procedure based on the ability of Pseudomonas organisms to oxidize p-aminodimethylaniline oxalate in the presence of molecular oxygen with the subsequent formation of indophenol blue within 10 to 30 seconds following the addition of

alpha naphthol. Steel (53) studied the oxidase activity among bacterial genera by employing a 1 percent (W/V) aqueous tetramethyl p-phenylenediamine dihydrochloride solution. Goldsberg and Armstrong (21) used p-aminodimethylaniline oxalate alone for identifying leptospiral colonies grown on Cox's agar medium. Kovacs (35) used tetramethyl p-phenylenediamine dihydrochloride solution for the identification of Pseudomonas pyocyanea.

V. DISTRIBUTION OF OXIDASE ACTIVITY AMONG BACTERIAL GENERA

Available evidence would indicate oxidase activity has appeared to be among the gram negative organisms. Steel (53) found that aerobic and facultative aerobic bacteria gave positive results whereas anaerobic bacteria gave negative, weak or doubtful reactions. Of 1660 strains tested by Steel (53), 210 gave positive and 64 gave delayed reactions. Delayed positive reactions were common in the general Bacillus, Brucella, Haemophilus and Pasteurella. All tested strains of the genus Pseudomonas were oxidase positive, with the exception of one strain of Pseudomonas ovalis, in which the reaction was delayed. All strains of Achromobacter anitratus were oxidase negative, but seven strains of Actinobacillus equali gave equal results. Strains of the genus Alcaligenes all gave positive reactions.

Gaby and Free (19) considered Kovac's (35) test to be oversensitive, because strains of Alcaligenes faecalis gave reactions similar to those of Pseudomonas aeuroginosa. All strains of the genus vibrio were oxidase positive, with a few exceptions. Of the 1660 strains tested by Steel (53), 744 were members of the Enterobacteriaceae, and none of these gave

a positive or even a delayed positive reaction. Neither was oxidase activity reported in any of the 1220 strains of Enterobacteriaceae tested by Ewing and Johnson (18).

A survey of literature has revealed a series of conflicting reports about the oxidase positive characteristics of certain microorganisms. Castell and Garrard (8) reported a positive reaction with the genus Alcaligenes, although Gaby and Hadley (20) and Hankin and Dillman (24) observed a negative reaction with the same genus. When the reaction was thought to depend on the presence of peroxide and peroxidase, Gordon and McLeod (22), Callow (6) and Ellingworth et al. (17) reported a positive reaction with certain species of the genus Bacillus. On the other hand, the investigations of Keilin (32), Gaby and Free (19) and Gaby and Hadley (20) indicated no significant oxidase activity in members of the genus Bacillus. Castell and Garrard (8) observed a positive reaction with Bacillus subtilis, Bacillus cereus, Bacillus graveolens and Bacillus mesentericus. A negative or doubtful reaction was observed in the genera Escherichia, Aerobacter and Proteus. A completely negative reaction was observed with the genera Clostridium, Streptococcus, Staphylococcus, Micrococcus and Lactobacillus.

The color development noticed upon flooding the plates with the oxidase reagent differed with the type of dye used. Castell and Garrard (8) observed a deep purple color with tetramethyl p-phenylenediamine hydrochloride. The central portion of the colony was stained deepest while the periphery of the colony was stained very light. The color developed appeared to be lost around the edges of the colony even among

the strong oxidase positive organisms. When a mixture of alpha naphthol and p-aminodimethylaniline oxalate were employed for the detection of oxidase activity among microorganisms, a blue color was noticed in positive cases indicating the synthesis of indophenol.

Kovacs (35) identified Pseudomonas pyocyanea by the oxidase reaction by placing a few drops of 1 percent tetramethyl p-phenylenediamine dihydrochloride solution on Whatman's No. 1 filter paper. The suspected colony was removed with a platinum rod and smeared on the reagent-impregnated filter paper in a line 3 to 6 mm long. The transferred colony turned dark purple in five to ten seconds in positive cases. The cytochrome oxidase test adopted by Gaby and Hadley (20) consisted of adding to a 12 to 18 hour nutrient broth (2 to 5 ml) a culture of the test organism, 0.3 ml of 1 percent aqueous solution of p-aminodimethylaniline oxalate and 0.2 ml of a 1 percent ethanol solution of alpha naphthol. The tube was shaken immediately to insure thorough oxygenation of the culture. A blue color was observed indicating the presence of cytochrome oxidase enzyme. Formation of an immediate blue color was attributed to the presence of Pseudomonas organisms while a slowly developing blue color was thought to indicate Alcaligenes faecalis. Hankin and Dillman (24) flooded the plates with the oxidase reagent at the end of an incubation period of 48 hours at 32 C. After 10 to 15 minutes, colonies turned blue in positive cases.

VI. IMPORTANCE OF OXIDASE REACTION

Gordon and McLeod (22) described the potential usefulness of the oxidase reaction in differential diagnosis of Gonococcus, on the one hand, and Staphylococcus and Streptococcus on the other. Previously, the oxidase test had been used mainly in clinical laboratories for the differential diagnosis of certain pathogenic bacteria, but Steel (53) employed the oxidase test as a taxonomic tool in microbiology. Callow (6) reported that "indophenol synthesis" by the oxidase reaction was of great value in demonstrating the distribution of myeloid tissue in normal foetal organs, and of granular leucocytes in the organs in myeloid leukemia. Because the oxidase test was a convenient method for identifying bacteria, Castell and Garrard opined that oxidase reaction should have a wider application, especially in the study of microbial spoilage of foods.

Recent investigations by several research workers (24,25,26,27) on application of the oxidase test have revealed a potential usefulness of this method in solving certain problems in the food industry. Hankin and Dillman (24) regarded the oxidase reaction as a rapid test to find "potentially" psychrophilic organisms in pasteurized dairy products. The rapid test was based upon the oxidase positive characteristic of Pseudomonas organisms. Based upon certain assumptions, the oxidase test was positively correlated with the standard test when applied to 133 samples of pasteurized bottled dairy products. Further applying the oxidase test to refrigerated delicatessen foods, Hankin and Ullman (26)

reported that the test provided additional information about the source of contamination in the sample, and expressed the belief that a comprehensive conclusion could be made when the total count was coupled with the results of the oxidase count and the coliform count.

Hankin et al. (25) reported the determination of oxidase positive organisms in raw milk samples was an effective means for indication of proper cleaning techniques and sanitary procedures used on the farm. The authors described the need of a quick method to assess whether the farmer was producing a "pseudomonad free" milk. Explanation with regard to the simplicity of the oxidase test was given leading to the establishment of certain standards in the quality control of milk production. Samples of raw milk have been suspected of psychrophilic contamination if the oxidase count was over 3,900 per ml, the percentage of oxidase positive organisms in the total count was over 16.5 and the Standard Plate Count was over 20,000 per ml. If a tested sample appeared positive to these standards, immediate remedial measures were suggested for sanitary practices at the farm. Upon following the recommended suggestions, the oxidase count in subsequent tests was reduced dramatically. Thus, the authors (25) concluded that a potential trouble spot was predicted by the oxidase test well before the detection by the standard test, and further regarded the oxidase test as "an additional tool in the never ending struggle to help provide quality food." Since Pseudomonas organisms are biochemically active, Hankin and Stephens (27) reported that the key to the prediction of keeping quality of refrigerated

foods was not the total bacterial count, but rather the measurement of the biochemical activity of organisms present in the sample.

VII. SPOILAGE ORGANISMS IN COTTAGE CHEESE

The quality of Cottage cheese is critical both for the processor and consumer, suggesting the need for a well established quality control program in the Cottage cheese industry. The nutrient quality of Cottage cheese makes an excellent medium for the growth of psychrophilic microorganisms making the product unacceptable for human consumption.

Many investigators (9,10,11,15,28,44,46,47) have reported the psychrophilic spoilage of Cottage cheese chiefly to the organisms of the genus Pseudomonas, Alcaligenes, Achromobacter, Proteus, Flavobacterium, Aerobacter and Escherichia. Yeast and molds also present another major problem to the keeping quality of Cottage cheese.

The various defects produced by these organisms include surface slime, surface discoloration, off flavors and off odors (3,10,39,46). Bonner et al. (4) isolated 17 organisms from spoiled Cottage cheese which were identified as two species of Achromobacter, one of Alcaligenes, two of Escherichia, three of Micrococci, and four of Pseudomonas.

Parket et al. (44) reported that three species of bacteria, Alcaligenes metalcaligenes, Pseudomonas viscosa and Pseudomonas fragi, were responsible for the production of a gelatinous or slimy curd defect in Cottage cheese. Though there was retarded development of the defect at low temperature and pH, these organisms were capable of producing the defect at initial pH values as low as 4.6 and at temperatures as low

as 3.5 C. The defect was accompanied by distinct off flavors. The characteristic of the defect produced has been described in detail by Elliker et al. (16) and Marth (39). Davis and Babel (10) reported slime formation by 15 cultures representing five genera (Achromobacter, Aerobacter, Alcaligenes, Proteus, and Pseudomonas). Experiments conducted at a temperature range of 4.4 C to 21 C revealed that the defect was more pronounced with a rise in temperature.

Studies of microbial flora of Cottage cheese have revealed wide ranges in bacterial count per gram of cheese. Overcast and Holt (42) observed that in 75 fresh samples obtained directly from manufacturers, coliform counts per gram of cheese ranged from 0 to 9,000, yeast and mold counts from 0 to 29,000 with total counts of less than 3,000 to 15,000,000, and psychrophilic counts from less than 3,000 to 12,000,000. The following counts were observed for the samples stored for ten days at 4 C: coliform counts per gram of cheese ranged from 0 to 680, yeast and mold counts from 0 to 23,000 with total counts of less than 3,000 to 1,600,000, and psychrophilic counts of less than 3,000 to 16,000,000. Similar observations were reported in studies conducted by other investigators (29,43).

Deterioration of Cottage cheese held at refrigerator temperatures can be prevented by following recommended sanitary practices during manufacturing, since bacterial spoilage is a major sales deterrent to the Cottage cheese industry. Once the growth initiates, the organisms grow very rapidly. The generation time of these organisms has a

tremendous impact on their rate of multiplication as reported by Overcast (41). Hence, every effort should be made to reduce the initial growth of these organisms in various refrigerated food products.

CHAPTER III

MATERIALS AND METHODS

I. BACTERIAL MEDIA

The dehydrated form of Standard Methods Agar (Baltimore Biological Laboratory, Baltimore, Maryland), recommended by Standard Methods for the Examination of Dairy Products (1), was selected for use and prepared in accordance with manufacturer's instructions.

II. PHOSPHATE BUFFER

A gelatin phosphate buffer used by Naylor and Smith (40) was employed in preparing the water blanks for determining the counts of Pseudomonas organisms from pure cultures. The gelatin phosphate diluent prepared was of the following composition: gelatin, 0.2 percent; monobasic sodium phosphate, 0.725 percent; dibasic sodium phosphate, 0.37 percent. The pH was adjusted to 6.5.

III. PURE CULTURES OF ORGANISMS

The oxidase reaction was observed among pure cultures of the following organisms usually encountered in refrigerated dairy products: Pseudomonas fragi, Pseudomonas fluorescens, Flavobacterium suaveolens, Alcaligens viscolactis, Bacillus cereus, Aerobacter aerogenes, and Escherichia coli. Stock culture of Bacillus cereus 14579 was obtained

from the American Type Culture Collection Center. The other cultures were obtained from the stock culture collection of the Microbiology Laboratory of the Food Technology and Science Department at The University of Tennessee.

IV. MAINTENANCE OF PURE CULTURES

Stock cultures of organisms were reconstituted with 3 to 5 milliliters of sterile Trypticase Soy Broth solution, which was prepared according to the specifications of the manufacturer. The reconstituted materials were transferred to sterile Trypticase Soy Broth tubes and were incubated at their optimum temperatures. After 24 hours of incubation, the cultures were transferred to fresh sterile tubes containing Trypticase Soy Broth and incubated at their optimum temperature. Similarly, the third transfers were made from which a 16-hour old culture was streaked on Trypticase Soy Agar slants. After observing the growth at the end of 24 to 48 hours, these agar slants were stored at 7 C. The cultures from these agar slants were inoculated in tubes containing sterile Trypticase Soy Broth and incubated at their optimum temperatures. These actively growing young cultures were plated at the end of 16 hours of incubation. The plating technique followed the specifications supplied in Standard Methods for the Examination of Dairy Products (1).

V. PREPARATION OF OXIDASE REAGENT

The oxidase reagent was prepared in accordance with the procedure used by Hankin and Dillman (24). A 1 percent solution of alpha naphthol

in 95 percent ethanol and a 1 percent aqueous solution of p-aminodimethyl-aniline oxalate were prepared separately. To dissolve the dye, the solution was heated gently and then cooled to room temperature in a cold water bath before being mixed with the alpha naphthol solution. The reagent was protected from light by keeping in an amber-colored bottle and was stored at 7 C. Fresh reagent was used throughout the study to avoid any misleading results caused by instability of the reagent.

VI. SAMPLES USED

Seventy-five samples of Cottage cheese secured from manufacturers and retail outlets throughout Tennessee over a period of six months were observed for total counts, oxidase counts, psychrophilic counts and an oxidase count of the psychrophilic colonies.

VII. PREPARATION OF SAMPLES FOR ANALYSES

A Virtis "45" homogenizer manufactured by The Virtis Company, Inc., Gardiner, New York, was used to blend the Cottage cheese samples. The following procedure was used for preparing samples:

1. Ten grams of a representative sample of cheese was weighed in a sterile 100 milliliter homogenizer stainless steel flask.
2. Ten milliliters of sterile 2 percent sodium citrate solution were added to the container to disperse and dilute the Cottage cheese curd.

3. The sample was blended with the Virtis homogenizer for one minute at a setting of 25.
4. The flask was removed from the homogenizer and the sample was ready to plate.

The samples emulsified with the above procedure exhibited a homogeneous, creamy consistency, with no large unbroken curd particles. All parts of the homogenizer coming in contact with the cheese were sanitized thoroughly by boiling prior to preparation of the samples.

VIII. MICROBIOLOGICAL EXAMINATION

Appropriate dilutions were made and plating was done for Standard Plate Count and psychrophilic counts.

Psychrophilic Plate Count

Methods as outlined in "Standard Methods for the Examination of Dairy Products" (1) were followed for obtaining the psychrophilic count. Plates were incubated at 7 C for ten days.

Standard Plate Count

Plates were incubated at 32 C for 48 hours as prescribed in Standard Methods for the Examination of Dairy Products (1).

Oxidase Count of the Standard Plate Count

The procedure used by Hankin and Dillman (24) was followed for obtaining the oxidase count. After determining the total count, the same plates were used to obtain the oxidase count. The plates were

flooded with a few milliliters of the oxidase reagent described previously and a reaction time of 10 to 15 minutes was allowed for the reagent to diffuse through the agar. The plates were then recounted for blue oxidase positive colonies. In some cases, the intensity of color from the colony counter was diminished by placing a piece of tissue paper between the light source and the bottom of the petri dish. This was useful for better evaluation of the plate, and particularly for observing the color development of subsurface and weak positive colonies.

Oxidase Count of the Psychrophilic Plate Count

After obtaining the psychrophilic count, each plate was flooded with the oxidase reagent to determine whether oxidase positive organisms were present.

IX. STATISTICAL ANALYSIS

Statistical analysis by paired t test was applied as outlined by Snedecor (52).

CHAPTER IV

RESULTS AND DISCUSSION

Of 75 samples of Cottage cheese analyzed during a period of six months, only 33 samples were included for statistical analysis of the data. The data of the remaining 42 samples are not presented because the samples contained less than 3,000 organisms per gram of cheese. The means for the Standard Plate Count, oxidase count of the Standard Plate Count, Psychrophilic Plate Count and an oxidase count of the Psychrophilic Plate Count are shown in Table I.

I. OXIDASE REACTION AMONG PURE CULTURES OF ORGANISMS

Oxidase test applied to some of the common organisms encountered in refrigerated dairy products revealed whether they are oxidase positive. A strong oxidase positive reaction was noted with pure cultures of Pseudomonas fragi and Pseudomonas fluorescens. The color of the colonies was deep blue. A positive reaction was noted with Flavobacterium suaveolens. A negative reaction was observed among Alcaligenes viscolactis and Bacillus cereus. The most superficial colonies of Bacillus cereus on an agar plate took on a light blue color which disappeared after a few minutes. Aerobacter aerogenes and Escherichia coli were completely negative for the oxidase test.

Data pertaining to the oxidase reaction among pure cultures of Pseudomonas fragi and Pseudomonas fluorescens are presented in

TABLE I

STANDARD PLATE COUNT, OXIDASE COUNT OF THE STANDARD PLATE COUNT, PSHCHROPHILIC PLATE COUNT AND OXIDASE COUNT OF PSHCHROPHILIC PLATE COUNT OF COTTAGE CHEESE SAMPLES

Sample No.	SPC[a]	Oxidase Count of SPC	PPC[b]	Oxidase Count of PPC
	millions			
1	0.0094	0.0025	0.1860	---[c]
2	1.5400	1.3000	3.2000	0.5000
3	1.3100	0.9000	2.2300	0.3500
4	0.0430	0.0100	0.0096	---
5	0.1270	0.0910	0.2200	0.0400
6	0.1050	0.0350	0.2100	0.0350
7	0.2880	0.0200	0.1200	0.0300
8	0.9500	0.3000	0.9000	0.0200
9	0.5000	0.2000	0.5500	0.1500
10	0.4500	0.1500	0.4800	0.1200
11	0.3300	0.1000	0.3600	0.1000
12	0.2250	0.0950	0.2300	0.0500
13	0.4300	0.1700	0.4650	0.1000
14	0.6700	0.2000	0.6000	0.1000
15	0.5300	0.3500	0.5500	---
16	0.7400	0.3600	0.7000	0.2000
17	0.3500	0.1200	0.4000	0.1000
18	0.1750	0.0500	0.1800	---
19	1.0600	0.4000	1.1000	---
20	0.1720	---	0.2300	0.0200
21	1.1300	0.7500	1.5000	0.4000
22	2.3400	0.2800	1.1500	---
23	1.2100	0.1300	1.2500	---
24	0.2210	0.1100	0.2400	0.0300
25	0.5100	0.1600	0.5500	0.1500
26	0.0480	0.0030	0.0420	---
27	3.5000	0.9000	4.0000	1.0000
28	2.5100	0.9500	2.6000	0.5000
29	2.5800	1.0900	2.4800	0.5000
30	0.2180	0.0640	0.2200	---
31	0.4400	0.0400	0.6000	0.1500
32	0.2650	0.1210	0.2400	0.0900
33	0.0138	0.0068	0.0066	0.0045

[a]Standard Plate Count.

[b]Psychrophilic Plate Count.

[c]Negative for oxidase test.

Tables II and III. The mean oxidase count of the Standard Plate Count was slightly higher than the mean Standard Plate Count. This occurred because Pseudomonas organisms form pin-point colonies which cannot be enumerated under ordinary conditions, but when they turned blue in the oxidase test, they were easily visible. This observation was in agreement with the work of Hankin and Dillman (24). Analysis of the data with paired t test among pure cultures of Pseudomonas organisms revealed a significant difference at 5 percent level of probability between the oxidase test and the standard procedure (1). However, they were not significantly different at 1 percent level. The mean Psychrophilic Plate Count was slightly higher than the mean oxidase count of the Standard Plate Count. This was because all Pseudomonas organisms do not grow well at 32 C incubation, but they can grow readily at refrigerated temperature.

After taking the Psychrophilic Plate Count, these plates were flooded with the oxidase reagent to confirm whether all colonies showed the oxidase positive characteristic. The data indicated that over 90 percent of Pseudomonas colonies on these plates were oxidase positive. The decrease in number probably occurred because the colonies with no marked development were very weak reactive and hence were not counted. The reaction with these pure cultures indicated that the oxidase count of Pseudomonas fragi and Pseudomonas fluorescens which were reported to be the most common dairy psychrophiles (41), gave a true reflection of Psychrophilic Plate Count.

TABLE II

STANDARD PLATE COUNT, OXIDASE COUNT OF STANDARD PLATE COUNT, PSYCHROPHILIC PLATE COUNT AND OXIDASE COUNT OF THE PSYCHROPHILIC PLATE COUNT OF A PURE CULTURE OF PSEUDOMONAS FRAGI

Replications	SPC[a]	Oxidase Count of SPC	PPC[b]	Oxidase Count PPC
	millions			
1	74	72	73	73
2	160	160	162	152
3	178	178	205	185
4	82	82	88	65
5	160	158	168	162
6	75	74	72	72
7	116	110	102	102
8	44	46	45	45
9	36	35	34	32
10	185	215	235	215
11	52	55	62	61
12	43	47	59	59
13	56	56	53	35
14	56	45	55	42
15	189	184	187	180

[a]Standard Plate Count.

[b]Psychrophilic Plate Count.

TABLE III

STANDARD PLATE COUNT, OXIDASE COUNT OF THE STANDARD PLATE COUNT, PSYCHROPHILIC PLATE COUNT AND OXIDASE COUNT OF THE PSYCHROPHILIC PLATE COUNT OF A PURE CULTURE OF PSEUDOMONAS FLUORESCENS

Replications	SPC[a]	Oxidase Count of SPC	PPC[b]	Oxidase Count of PPC
	millions			
1	42	42	38	38
2	39	39	40	40
3	43	40	40	36
4	226	228	290	240
5	42	48	53	52
6	35	34	32	32
7	31	32	30	28
8	32	32	31	31
9	31	31	30	30
10	64	68	64	60
11	70	68	65	62
12	60	60	61	61
13	108	110	115	95
14	35	31	43	40
15	51	45	55	49

[a]Standard Plate Count.

[b]Psychrophilic Plate Count.

The data pertaining to the oxidase reaction of Flavobacterium suaveolens are presented in Table IV. This organism was positive for the oxidase test and did not grow at refrigerator temperature, hence Psychrophilic Plate Count was not reported. The Standard Plate Counts of Alcaligenes viscolactic, Bacillus cereus, Aerobacter aerogenes and Escherichia coli were found to be negative for the oxidase test, did not grow at refrigerator temperatures, and Psychrophilic Plate Counts were negative.

II. OXIDASE REACTION AMONG MICROBIAL FLORA OF COTTAGE CHEESE

All 75 samples of Cottage cheese were analyzed to determine whether the oxidase count of the Standard Plate Count was a true reflection of the Psychrophilic Plate Count obtained by the Standard Method (1).

The samples tested included fresh samples secured from manufacturers, retail store samples, and the samples stored for ten days. Most of the fresh samples contained total counts of less than 3,000 organisms per gram of cheese. Those samples with total counts of less than 3,000 were negative for the oxidase count and Psychrophilic Plate Count, and hence have not been included in the analysis of the data.

After determining the Standard Plate Counts, the same plates were flooded with the freshly prepared oxidase reagent previously described. The oxidase count was obtained, after allowing a reaction time of 10 to 15 minutes, by counting only those colonies which turned blue. The color reaction observed ranged from deep blue to light blue among the positive colonies. Colonies stained very light were omitted from enumerating to

TABLE IV

STANDARD PLATE COUNT AND OXIDASE COUNT OF THE STANDARD PLATE COUNT OF FLAVOBACTERIUM SUAVEOLENS

Replications	SPC[a]	Oxidase Count of SPC
	millions	
1	3.5	3.2
2	3.1	3.0
3	8.8	8.1
4	8.7	7.2
5	3.2	3.1
6	7.3	7.2
7	15.2	14.2
8	12.0	11.2
9	3.2	2.8
10	3.5	2.6
11	3.1	2.6
12	11.0	9.5
13	11.5	7.6
14	8.8	7.6
15	8.7	8.2

[a]Standard Plate Count.

avoid any misleading results. Observations revealed that only 38 percent of the organisms of the total count were oxidase positive.

Available literature (3,10,15,37,44) have reported the Pseudomonas organisms as the microbial flora predominant in the spoilage of Cottage cheese. Since these organisms are strongly oxidase positive their numbers would be expected to be reflected in the oxidase count. This, however, was not true in the present investigation. Therefore, these organisms may not constitute the major microbial flora of Cottage cheese to the extent of 90 percent or higher. In fact, for Cottage cheese to become unacceptable by the consumer, it may not necessarily have a high count of Pseudomonas organisms. The biochemical activity of these organisms can also bring about a change in the product rendering it unacceptable for human consumption. This belief was also expressed by Hankin and Stephens (27), who reported that the keeping quality of refrigerated food products does not necessarily depend on the total count of microorganisms, but rather the biochemical activity of those present.

An interesting observation was noted with sample number 20 (Table I, page 24), in which case the total count was 172,000 per gram of cheese and negative for the oxidase test. Surprisingly, the Psychrophilic Plate Count was 230,000. This observation gave rise to a question of whether a single Pseudomonas species was present. However, confirmation by isolation and identification of colonies, was not made since that was not a part of the present investigation. One sample which was not included in the data analyzed statistically contained

285,000 organisms on Standard Plate Count and was negative for both the oxidase test and Psychrophilic Plate Count. This observation suggested that the oxidase test revealed a true reflection of the Psychrophilic Count because of the perfect coincidence of the oxidase test with the Standard test. However, a conclusion to any extent cannot be made with this single observation because of the presence of a larger significant difference observed between these two methods in the present investigation (Table V). This observation, however, raised a question as to what type of organisms were present.

The mean total count was over 760,000 organisms per gram of Cottage cheese (Table VI). The mean oxidase count determined in the Standard Plate Count plates was over 290,000 and the percentage of oxidase positive organisms in the total count was 38. After taking the Psychrophilic Plate Count, each plate was flooded with the oxidase reagent to determine what percentage of the psychrophilic count showed oxidase positive characteristic. These data revealed that only 17 percent of the organisms of the Psychrophilic Plates were oxidase positive. The mean of Psychrophilic Plate Count was higher than the mean of the Standard Plate Count, and the mean oxidase count of the Psychrophilic Plate Count should be higher than the mean oxidase count of the Standard Plate Count. Some of the psychrophilic plates with more than one million psychrophilies per gram of cheese were negative for oxidase test. These observations created doubt that the oxidase test was giving a true representation of the psychrophilic count. Besides, the literature revealed a series of conflicting reports about the oxidase positive

TABLE V

PAIRED t ANALYSIS BETWEEN OXIDASE COUNT OF THE STANDARD PLATE COUNT AND PSYCHROPHILIC PLATE COUNT OF COTTAGE CHEESE SAMPLES, AND PURE CULTURES OF PSEUDOMONAS ORGANISMS

Source	$\bar{d}$	$\Sigma(d-\bar{d})^2$	s^2_d	$s_{\bar{d}}$	t
Cottage cheese samples	-0.556	14.329	0.447	0.116	-4.793***
Pure cultures of Pseudomonas organisms	-5.400	4967.200	171.280	2.388	-2.260*

* Statistical significance at the 0.05 level of probability.

***Statistical significance at the 0.001 level of probability.

TABLE VI

MEAN PLATE COUNTS AND PERCENTAGES OF OXIDASE POSITIVE ORGANISMS IN COTTAGE CHEESE SAMPLES AND PURE CULTURES OF PSEUDOMONAS ORGANISMS

Source	Count	SPC[a]	Oxidase Count of SPC	PPC[b]	Oxidase Count of PPC
Cottage cheese	Mean[c]	760,000	290,000	840,000	143,000
	Percent of oxidase positive organisms	38.15	---	17.02	---
Pure cultures of Pseudomonas organisms	Mean[d]	80,500,000	80,800,000	86,000,000	79,000,000
	Percent of oxidase positive organisms	100.37	---	91.00	---

[a]Standard Plate Count.

[b]Psychrophilic Plate Count.

[c]Mean of 33 observations.

[d]Mean of 30 observations.

characteristic of the genus Alcaligenes (8,19,24,53). By the same token, organisms of the genus Proteus have also been reported as oxidase negative (8,53). If these organisms are classified as psychrophiles, and if they are not oxidase positive, then the oxidase count may not give a true representation of the psychrophilic count.

Should the oxidase test be substituted solely for the standard procedure for determining psychrophiles (1) on the basis of a conclusion that no significant difference should be observed between the oxidase count and Psychrophilic Plate Count, such a conclusion may be erroneous because all psychrophilic microorganisms are not necessarily oxidase positive, and apparently, not all Pseudomonas organisms involved in spoilage of Cottage cheese are capable of showing the oxidase positive characteristic.

The present investigation indicates that if the Standard Plate Count is positive for oxidase count, that particular sample may be considered as positive for psychrophiles. Hence, setting certain limits of standards for oxidase positive organisms might help in applying quick remedial measures.

Though the presence of Pseudomonas organisms as a predominant microflora of Cottage cheese has not been doubted, this study gave an impression that Pseudomonas organisms are not composing the vast majority of the spoilage organisms in Cottage cheese. Also, the incubation temperature of the Psychrophilic Plate Count and microenvironment in the cheese may have a deleterious effect on the concentration of

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cytochrome oxidase enzyme system of Pseudomonas organisms involved in the spoilage of Cottage cheese. These two statements possibly then could account for the failure of the oxidase test to correlate with the Psychrophilic Plate Count.

CHAPTER V

SUMMARY AND CONCLUSIONS

Samples of Cottage cheese secured from manufacturers and retail stores in Tennessee over a period of six months were analyzed to determine whether the oxidase count of Standard Plate Count was a true reflection of psychrophilic count obtained by the Standard procedure. A preliminary study on some on the pure cultures of microorganisms important to the dairy industry revealed Pseudomonas organisms were strongly oxidase positive.

The mean total count of 33 Cottage cheese samples was over 760,000 organisms per gram (Table VI). The mean oxidase count was 290,000, constituting 38 percent of the total count. The mean Psychrophilic Plate Count was 840,000 whereas, the mean oxidase positive organisms on the psychrophilic plates was 143,000 constituting only 17 percent. Some of the psychrophilic plates having more than one million psychrophiles per gram of cheese were negative for oxidase test. Oxidase test and the standard test were significantly different at any given level of probability with paired t analysis of the data (Table V).

Based on the results of this investigation, the following conclusions were reached:

1. The oxidase test did not give a true reflection of psychrophilic microorganisms involved in spoilage of Cottage cheese.

2. The oxidase test gave rise to the question whether all *Pseudomonas* organisms involved in the spoilage of Cottage cheese are capable of showing the oxidase positive characteristic.

LIST OF REFERENCES

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LIST OF REFERENCES

1. American Public Health Association. 1972. Standard Methods for the Examination of Dairy Products, 13th ed. Amer. Public Health Assoc., Washington, D. C.

2. Baumann, D. P., and G. W. Reinbold. 1963. Enumeration of psychrophilic microorganisms. J. Dairy Sci., 26:81.

3. Bonner, M. D., and L. G. Harmon. 1957. Characteristics of organisms contributing to spoilage in Cottage cheese. J. Dairy Sci., 40:1599.

4. _____, and C. K. Smith. 1957. Defects produced by spoilage organisms in Cottage cheese sterilized by cathode rays. J. Dairy Sci., 40:1363.

5. Brandenburg. 1960. Munchenmed. Wchnschr., S. 183. Cited by Dunn, J. S. 1911. The oxidase reaction in myeloid tissues. J. Path. Bact., 15:20.

6. Callow, A. B. 1926. The heat stable peroxidase of bacteria. Biochem. J., 20:247.

7. Carpenter, C. M., L. G. Suhrland, and M. Morrison. 1947. The oxalate salt of p-aminodimethylaniline, an improved reagent for the oxidase test. Science., 105:649.

8. Castell, C. H., and E. H. Garrard. 1940. "Oxidase reaction" of bacteria in relation to dairy products. Food Res., 5:215.

9. Collins, E. B. 1955. Factors involved in the control of gelatinous curd defects of Cottage cheese. I. Storage temperature and pH. J. Milk Food Technol., 18:169.

10. Davis, P. A., and F. J. Babel. 1954. Slime formation on Cottage cheese. J. Dairy Sci., 37:176.

11. Deane, D., F. E. Nelson, and R. W. Baughman. 1954. Microbial flora and keeping quality of packaged Cottage cheese. Am. Milk Rev., 16:60.

12. Dietrich and Liebermeister. 1902. Sauerstoffubertragende Kornchen in Milzbrandbacillen. Central. f. Bakt. Orig., 32:858. Cited by Gordon, J., and J. W. McLeod. 1928. The practical application of the direct oxidase reaction in bacteriology. J. Path. Bact., 31:185.

13. Doetsch, R. N., and W. M. Scott. 1951. Psychrophilic bacteria in milk and cream. Milk Plant Monthly, 40:30.

14. Dunn, J. S. 1911. The oxidase reaction in myeloid tissues. J. Path. Bact., 15:20.

15. Elliker, P. R. 1954. Fine points of sanitation that up Cottage cheese keeping quality. Food Eng., 26:79.

16. _____, E. L. Singh, L. J. Christensen, and W. E. Sandine. 1964. Psychrophilic bacteria and keeping quality of pasteurized dairy products. J. Milk Food Technol., 27:69.

17. Ellingworth, S., J. W. McLeod, and J. Gordon. 1929. Further observations on the oxidation by bacteria of compounds of the para-phenylenediamine series. J. Path. Bact., 32:173.

18. Ewing, W. H., and J. G. Johnson. 1960. The differentiation of Aeromonas and C_{27} culture from interobacteriaceae. Int. Bull. Bact. Nom. Tax., 10:223.

19. Gaby, W. L., and E. Free. 1958. Differential diagnosis of Pseudomonas-like microorganisms in the clinical laboratory. J. Bacteriol., 76:442.

20. _____, and C. Hadley. 1957. Practical laboratory test for the identification of Pseudomonas aeruginosa. J. Bacteriol., 74:356.

21. Goldsberg, H. S., and J. C. Armstrong. 1959. Oxidase reaction with Leptospiral colonies and its adaptations to antibiotic sensitivity testing. J. Bacteriol., 77:512.

22. Gordon, J., and J. W. McLeod. 1928. The practical application of the direct oxidase reaction in bacteriology. J. Path. Bact., 31:185.

23. Greene, V. W., and J. J. Jezeski. 1954. Influence of temperature on the development of several psychrophilic bacteria of dairy origin. Appl. Microbiol., 2:110.

24. Hankin, L., and W. F. Dillman. 1968. A rapid test to find "potentially" psychrophilic organisms in pasteurized dairy products. J. Milk Food Technol., 31:141.

25. _____, A. R. Permice, and W. F. Dillman. 1968. Quality control of milk production by means of the cytochrome oxidase test. J. Milk Food Technol., 31:165.

26. Hankin, L., and W. Ullman. 1969. Application of oxidase test to refrigerated delicatessen foods. J. Milk Food Technol., 32:122.

27. _____, and G. R. Stephens. 1972. What tests usually predict keeping quality of perishable foods. J. Milk Food Technol., 35:574.

28. Harmon, L. G. 1963. Cottage cheese problems in production and sanitation. J. Milk Food Technol., 26:86.

29. _____, and C. K. Smith. 1956. The influence of environment and processing on spoilage organisms in Cottage cheese. J. Milk Food Technol., 19:252.

30. Holland, R. F. 1967. Psychrophiles: danger rises. Am. Dairy Rev., 29:42.

31. Ingram, J. L., and J. L. Stokes. 1959. Psychrophilic bacteria. Bact. Rev., 23:97.

32. Keilin, D. 1925. On cytochrome, a respiratory pigment common to animals, yeast and higher plants. Proc. Roy. Soc. (Lond), B. 98:312.

33. _____, and E. F. Hartsee. 1938. Cytochrome a and cytochome oxidase. Nature., 141:870.

34. _____. 1939. Cytochrome and cytochrome oxidase. Proc. Roy. Soc. (Lond) B. 127:167.

35. Kovacs, N. 1956. Identification of Pseudomonas pyocyanea by the oxidase reaction. Nature., 178:703.

36. Kramer. 1912. Beitrange zum Lefortigen Nachweis Von oxidations and reduktionswirk ungen der bakterien anf grund der neun methode Von W. H. Schultze. Central. f. Bakt. Orig., 62:394.

37. Kristoffersen, T., and B. M. Chakraborty. 1964. Keeping quality of creamed Cottage cheese. J. Dairy Sci., 47:931.

38. Lenhoff, H. W., and N. O. Kaplan. 1953. A cytochrome peroxidase from Pseudomonas fluorescens. Nature., 172:730.

39. Marth, E. H. 1969. Spoilage of Cottage cheese by psychrophilic microorganisms. Cult. Dairy Prod. J., 5:14.

40. Naylor, H. B., and P. A. Smith. 1946. Factors affecting the viability of Serratia Marcescens during dehydration and storage. J. Bacteriol., 52:565.

41. Overcast, W. W. 1967. Extending shelf-life by controlling psychrophiles. Am. Dairy Rev., 29:42.

42. _____, and H. Holt. 1972. Personnel communication. Food Technology and Science Dept., Univ. of Tenn., Knoxville.

43. _____, and J. D. Skean. 1958. Cottage cheese needs carefully controlled manufacturing and marketing practices. Tenn. Farm and Home Sci., Progress Report No. 27.

44. Parker, R. B., V. N. Smith, and P. R. Elliker. 1951. Bacteria associated with a gelatinous or slimy curd defect of Cottage cheese. J. Dairy Sci., 34:887.

45. Peterson, A. C., and M. F. Gunderson. 1960. Role of psychrophilic bacteria in frozen food spoilage. Food Technol., 14:413.

46. Reidy, G., and T. I. Hedrick. 1968. Cottage in U. S. A. III. Quality control to continuous manufacturer. Dairy Indus., 33:617.

47. _____. 1968. Cottage cheese in U. S. A. III. Quality control. Dairy Indus., 33:531.

48. Schultz, W. H. 1910. Uebereine newe methode zum nachweis von reduktions and oxdationswirkungen der bacterien. Central. f. Bakt. Orig., 56:544.

49. Smith, L. 1954. Bacterial cytochromes. Bact. Rev., 18:106.

50. _____. 1954. An investigation of cytochrome c oxidase activity in bacteria. Arch. Bichem. Biophys., 50:315.

51. _____, and E. Stotz. 1954. Purification of cytochrome c oxidase. J. Biol. Chem., 209:819.

52. Snedecor, G. W. 1956. Statistical Methods, 5th ed. p. 45. Iowa State College Press, Ames, Iowa.

53. Steel, K. J. 1961. Oxidase reaction as a taxonomic tool. J. Gen. Microbiol., 25:297.

54. Vernon, L. P. 1953. Cytochrome c content of Rhodospirillum rubrum. Arch. Biochem. Biophys., 43:492.

55. Witter, L. D. 1961. Psychrophilic bacteria. A review. J. Dairy Sci., 44:983.

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