EFFECTS OF SELECTED PROTEOLYTIC BACTERIA ON CHEDDAR CHEESE RIPENING

by

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1. INTRODUCTION

Radiation dose is the term used for the actual radiation energy absorbed per unit mass of an absorbing medium. It is one of the physical quantities, like temperature, pressure, etc., that is measured indirectly. It is not the radiation dose itself but it is the effect of radiation dose on certain substances which is measured to assess the amount of total dose. Accordingly the definitions of the units of radiation dose are also based upon the effects of radiation dose, like for example the unit of exposure dose, 1 roentgen, is defined as "the quantity of gamma radiation which will produce, by ionization, one electrostatic unit of electricity of either sign in 1 cm^3 of dry air, measured at standard conditions of temperature and pressure", and the unit of absorbed dose in a material, 1 rad, is defined as the "absorbed energy of 100 erg per gram of material."

The art of measurement of radiation dose is known as radiation dosimetry. The need for appropriate dosimetry was recognized soon after the discovery of ionizing radiation. The radiation hazard involved in the use of ionizing radiation and its controlled use in biology, industry, medicine, research and military applications required accurate measurement of the radiation energy absorbed. Various dosimetry systems utilizing various effects of ionizing radiation on materials were developed. Early dosimetry devices consisted of ionization chambers coupled with an electrometer and photographic emulsions[1]. Then "chemical dosimetry" was developed during the years 1948 -

was not shortened.

The agents of Cheddar cheese ripening are the enzymes which gain entry into the cheese through the cheese milk, rennet extract, and lactic starter culture. It was first thought that the milk and rennet enzymes were the most important cheese ripening agents, but recently the endocellular enzymes, released by the autolysis of the bacteria present in cheese, have been shown to be the main source of active enzyme in Cheddar cheese.

A project involving the use of added bacterial enzyme systems as a means of acceleration of Cheddar cheese ripening has been undertaken at the Kansas Agricultural Experiment Station. The procedures employed and the results obtained in the early phases of the study are reported in this manuscript.

REVIEW OF LITERATURE

Cheddar Cheese Flavor Development

Hewitt, et al. (34) believed that the flavor of biological material was the result of mixtures of chemical compounds produced by the normal metabolic processes occurring within the material. Kosikowski's (42) "Theory of Component Balance" agrees with this general observation. He stated that typical Cheddar cheese flavor was a definite combination of several biological products, such as amino acids, fatty acids, alcohols,

esters, etc. Each of these compounds may or may not have their own individual flavor, but in proper ratio to each other and in proper concentration in the mass of cheese, these compounds have the typical flavor of Cheddar cheese according to Kosikowski.

The importance of peptides in Cheddar cheese flavor development was indicated by Storgards and Lindquist (74) and Pette (58). Storgards and Lindquist found that the concentrations of the various peptides differed between the various types of cheese. Furthermore they observed that the concentrations of the peptides varied between good and poor flavored cheese of the same variety. Bullock and Irvine (11) identified 18 amino acids from aged Cheddar cheese and they suspected the presence of other amino acids in minute quantities. Amino acids were observed by Kosikowski (41) in Cheddar cheese two days after its manufacture. He found that the variety and concentration of amino acids present in Cheddar cheese increased as the cheese ripened.

An aqueous solution of pure amino acids, in the same proportions as found in six month old Cheddar cheese, was prepared by Mabbitt (47). A brothy flavor occurred when he mixed the amino acid solution with bland fresh cheese curd. Mabbitt postulated that the brothy flavor may be a background flavor on which the typical flavor of Cheddar cheese was superimposed. Silverman and Kosikowski (67) reported that

they were successful in obtaining a cheese flavored compound by mixing pure amino acids and fatty acids in the same proportions as these acids occurred in aged Cheddar cheese. Individual amino acids were added to Cheddar cheese during its manufacture by Baker and Nelson (5). They observed that added histidine imparted a slightly bitter flavor to the resulting cheese and that serine yielded cheese with a slightly higher flavor than normal.

Silverman and Kosikowski (70,71) found that certain amines present in raw milk cheese were not present in pasteurized milk cheese. They also observed that all amines were present in greater concentrations in raw milk cheese than in pasteurized milk cheese. These workers explained these observations by postulating that the cheese bacterial flora controls the varieties and amounts of amines present in Cheddar cheese. A direct relationship between tyramine content and flavor intensity in Cheddar cheese was noted by Dahlberg and Kosikowski (18). Dacre (14) found very little correlation existing between these two values except for a general relationship as the cheese aged. Free hydrogen sulfide production was related to Cheddar cheese flavor development by Kristoffersen and Nelson (44). They observed that a high flavored cheese contained the most free hydrogen sulfide.

Babel and Hammer (4) stated that fat hydrolysis was beneficial to Cheddar cheese flavor production. The occurrence of

less butterfat hydrolysis in pasteurized milk cheese than in raw milk cheese was observed by Sheuring and Tuckey (66). Therefore, they postulated that the increased fat hydrolysis was an important factor in the development of the more pronounced cheese flavor present in the raw milk cheese. Dahlberg and Kosikowski (16) did not find a relationship existing between volatile fatty acid formation and Cheddar cheese flavor development in cheese. Silverman and Kosikowski (67) reported that a rancid flavor occurred when pure fatty acids were mixed in the same proportion as they occurred in aged Cheddar cheese. As cited previously, these workers obtained a cheese-like flavor from a mixture of fatty acids and amino acids.

Dacre (15) used gas-liquid chromotography to separate, into its component parts, a flavor distillate obtained from Cheddar cheese by steam distillation. The distillate possessed a cheese-like odor and was a neutral compound. Therefore, Dacre concluded that volatile fatty acids did not play an important role in Cheddar cheese flavor. Dacre identified several neutral compounds from the cheese distillate, including ethanol, butyraldehyde, ethyl acetate, ethyl butyrate, etc. He prepared aqueous solutions using pure forms of the identified compounds singly and in various combinations. He was unable to detect the odor or flavor of Cheddar cheese in any of these solutions.

Diacetyl was found to be present in Cheddar cheese throughout the entire ripening period by Calbert and Price (13). These

workers (12) thought that diacetyl, in small concentration, was important in Cheddar cheese flavor. Dacre (15) found small amounts of diacetyl present in Cheddar cheese up to three months of age, but he could not detect diacetyl in older, higher flavored cheese.

Evans (24) studied skim milk cultures of streptococci that produced volatile fatty acids and alcohols. She found that these acids and alcohols combined to form esters which she concluded were important cheese flavor components. Suzuki, et al. (75) supported her conclusion by steam distilling Cheddar cheese and obtaining a distillate which contained esters and had a cheese-like odor.

The Strecker degradation of amino acids to aldehydes was suggested by Keeney and Day (39) as a possible pathway of Cheddar cheese flavor development. They found that methional, obtained by Strecker degradation of methionine, had a definite Cheddar cheese-like odor in low concentrations. In separate work, Jackson (59) also obtained a Cheddar cheese-like-flavored aldehyde from methionine.

Proteolytic Enzyme Activity in Cheddar Cheese

Jackson (59) and Kosikowski (42) both stated that the agents which produce flavor in Cheddar cheese are enzymatic in nature and that these enzymes may come from the cheese milk, rennet extract, and bacterial flora in the cheese.

The role of milk proteinase in Cheddar cheese ripening was studied by Peterson, et al. (57). They found only a small amount of proteolytic activity present in the milk at the time it was placed in the cheese vat. Therefore, these investigators concluded that even if milk proteinase was active in the cheese environment, it would play a small role in the overall degradation of cheese protein.

The proteinases in rennet extract degrade casein to polypeptides and peptides, which are then broken down to amino acids and amines by bacterial peptidases (31,48,58). Sherwood (62) manufactured cheese from milk containing chloroform, to eliminate the action of bacterial proteinases on the cheese protein. He concluded that the peptones and peptides found in the cheese were end-products of rennet proteinase activity. Masayoshia (49) was not able to detect the liberation of amino acids by rennin acting on casein, however, he observed that peptides were released. Masayoshia also observed that pepsin, present in rennet extract as an impurity, released amino acids from casein. Berridge (8) manufactured separate lots of Cheddar cheese employing normal rennet extract, crystalline rennin, or crystalline rennin plus the impurities removed during crystallization of the rennin from rennet extract. He reported that crystalline rennin alone yielded a cheese lacking the flavor of normal cheese. The cheese, manufactured by using crystalline rennin plus its impurities,

had a flavor similar to the flavor of normal Cheddar cheese.

Peterson, et al. (57) observed that the first important increase in proteolytic activity in Cheddar cheese occurred in the first 90 to 120 minutes after the start of the manufacturing process. They believed that the activity was partly the result of proteinases added with the rennet extract, but that the activity was mainly due to the extracellular proteinases produced by the bacteria during the ripening of the cheese milk. These investigators also found that cysteine hydrochloride increased the activity of what they assumed to be endoproteinases of the bacterial flora in the cheese. The cysteine-activated proteolytic activity in cheese was observed by these workers after several days of storage and it was found to increase throughout the ripening period. Peterson, et al. (56) further observed that the proteolytic activity was always higher in raw milk cheese than in pasteurized milk cheese. They believed that this was the result of the reduced bacterial content of pasteurized milk Cheddar cheese. Whitehead and Lane (82) observed that penicillin, added to milk to eliminate bacterial growth in the resulting cheese, did not retard the ripening of Cheddar cheese. They concluded that the continued ripening could be attributed to endocellular enzymes liberated by autolysis of the bacterial cells present in the cheese. Freeman and

Dahle (30) stated that the rate of proteolysis in Cheddar cheese was directly related to the numbers and types of bacteria present in the cheese curd.

Peterson, et al. (57) found that casein was degraded by proteinases from cheese at an optimum pH of 5.0 with a secondary optimum occurring at pH 7.0 to 8.0. They believed that the activity observed at pH 5.0 represented endocellular proteinases released by bacterial autolysis, while that at pH 7.0 to 8.0 was the result of exocellular proteinases from the bacterial flora in the cheese. The enzyme systems of several species of bacteria isolated from Cheddar cheese were investigated by Baribo and Foster (6). They observed that of the enzyme systems investigated, all were active only at a neutral pH except one from Streptococcus lactis, which also possessed proteolytic activity at a pH of 5.0. Van der Zant and Nelson (79) found that cell-free extracts of S. lactis (S. hereafter designates Streptococcus) degraded casein at pH 5.5. These workers (78) previously had not observed proteolytic activity in the S. lactis culture media after removal of the bacterial cells by centrifugation. Therefore, Van der Zant and Nelson (79) concluded that the proteolytic activity of S. lactis was due to endocellular proteinases released upon autolysis of the cell. Brandsaeter and Nelson (9,10) observed that the proteinases and peptidases in cell-free extracts of Lactobacillus casei were active in the pH range

of Cheddar cheese ripening.

Lipolytic Enzyme Activity in Cheddar Cheese

Harper and Kristoffersen (31) stated that the lipolytic activity in Cheddar cheese may come from milk lipase, lipases added as impurities in the rennet extract, and bacterial lipases.

Milk lipase was observed to be inactive at a pH of 6.5 or lower by Peterson, et al. (53). Stadhoulders and Mulder (73) made the same observation plus the fact that milk lipase activity was greatly retarded by the salt concentrations that exist in Cheddar cheese. In conflicting work, Albrecht and Jaynes (1) found milk lipase to be active at pH 5.4. They employed lactic acid to lower the pH of the test substrate and commented that Peterson, et al. had used an inorganic acid which inactivated the milk lipase. Hart, et al. (33) made Cheddar cheese from milk which contained chloroform to prevent bacterial growth. They assumed that by eliminating bacterial growth, bacterial lipases would not be present in the cheese. These workers did not observe the production of fatty acids as the cheese aged and therefore concluded that the milk lipase was inactive. Peterson, et al. (55) later observed that less than 5 percent of the original milk lipase was present in Cheddar cheese. They found that the milk lipase was partially inactivated during the heating of the cheese curd

and that a large proportion of it was removed with the whey.

Lipolytic activity in Cheddar cheese was noted by Peterson, et al. (55) immediately after the addition of the rennet extract. They agreed with Stadhoulders and Mulder (73), and Harper and Kristoffersen (31) that the rennet extract may contain lipases as impurities. Peterson, et al. further noted that this activity was quickly dissipated and therefore would not be important in Cheddar cheese ripening. The subsequent lipolytic activity, observed by these workers, occurred after five to twenty days of storage. They also found that maximum activity occurred after 100 days of ripening. These investigators found that raw milk Cheddar cheese contained more total lipolytic activity than pasteurized milk Cheddar cheese. Peterson, et al. concluded that the lipases active in cheese ripening probably were endocellular lipases released by bacterial autolysis.

Peterson and Johnson (52) found that 12 whole milk cultures of lactobacilli and four whole milk cultures of micrococci exhibited lipolytic activity after 60 days, but not in 15 days of incubation. They believed that this activity was the result of endocellular lipases being released from the bacterial cells by autolysis.

Bacterial Flora of Cheddar Cheese

Harper and Kristoffersen (31) stated that very low bacterial count milk does not contain the "desirable" types of organisms needed to produce quality Cheddar cheese. They also stated that high bacterial count milk usually contains some of the "undesirable" types of organisms. In a very extensive study, Smith, et al. (72) found that high bacterial count milk was correlated with low flavor scores in the resulting cheese.

The bacterial flora of both raw and pasteurized milk Cheddar cheese was investigated by Evans, et al. (25). They found that raw milk cheese contained four main groups of bacteria, Bacterium lactic acidi (S. lactis), Bacterium casei (L. casei) (L. hereafter designates Lactobacillus), streptococci, and micrococci. They further observed that the bacterial flora of pasteurized milk cheese consisted mainly of starter culture organisms at first, but that it became more like the bacterial flora of raw milk cheese as it aged. These investigators did not observe that a higher proportion of any single group of bacteria was associated with a higher-flavored Cheddar cheese.

Because of the large number of non-lactic bacteria present in raw milk, Alford and Frazier (2) believed that this group of bacteria was an important factor in the higher flavor development in raw milk cheese than in pasteurized milk cheese

of the same age. Dawson and Feagen (21) used S. lactis, Streptococcus cremoris, and Streptococcus diacetilactis in separate starter cultures for making Cheddar cheese. They observed that all three organisms reached maximum numbers in the resulting cheese during the cheddaring process and that they decreased in numbers quickly as the cheese aged. These investigators believed that secondary organisms would have a favorable environment for growing in ripening Cheddar cheese.

Raw milk cheese was made by Davis (20) from high quality milk, employing a starter containing S. lactis and S. cremoris. He observed that these organisms were dominant in the cheese after one month of storage. Davis further observed that Streptobacterium casei (L. casei) was the only organism present in the cheese after five months of ripening. Sherwood (62) believed that the lactobacilli were important in Cheddar cheese flavor development. He found that Streptobacterium plantarum (Lactobacillus plantarum) was the dominant organism in young Cheddar cheese. Sharpe (60) observed lactobacilli in cheese one week after its manufacture. She further observed that these organisms dominated the cheese bacterial flora after two months of ripening.

Kristoffersen and Nelson (43) found that L. casei could utilize serine as a source of energy. They further observed that L. casei could not utilize phosphoserine. These workers

stated that the phosphatase present in raw milk would liberate serine from its phosphate form. From these observations, Kristoffersen and Nelson postulated that the inactivation of phosphatase by pasteurization and the subsequently reduced energy source for L. casei may be the reason why pasteurized milk Cheddar cheese does not develop flavor as fast as raw milk Cheddar cheese.

Mabbitt and Zielinska (48) were able to detect trace amounts of lactose and/or galactose in aged Cheddar cheese. They believed that these sugars would be a source of energy for the lactobacilli in cheese. Suzuki, et al. (75) did not detect the presence of milk sugar in cheese after three days of storage. Fagen, et al. (26) found milk sugar present in pasteurized milk cheese after 53 days of storage.

Attempts to Improve Cheddar Cheese Ripening

Evans, et al. (25) found that a mixed culture of Bacterium lactic acidi (S. lactis), streptococci, and micrococci, used in place of the regular starter culture in Cheddar cheese manufacture, gave a more desirable flavored cheese than did a pure culture of Bacterium lactic acidi (S. lactis). Evans (24) used a mixed culture of S. lactis and other streptococci as the cheese starter and obtained a cheese which had a higher flavor than normal cheese. She did not observe any bitterness

in the cheese.

Dahlberg and Kosikowski (17) used a pure culture of Streptococcus faecalis as the starter culture when making Cheddar cheese. They obtained a cheese that had good flavor and which did not develop any off-flavor after seven months of ripening. In a later study, these workers (19) concluded that the use of S. faecalis was feasible and desirable only when using low bacterial count milk for making Cheddar cheese. They stated that the milk normally employed for cheese making contained enough lactobacilli to give the cheese the desired flavor.

Using pure bacterial cultures in addition to the normal starter culture when manufacturing Cheddar cheese, Hucker and Marquardt (35) observed that Streptococcus citrovorus did not improve the flavor of the cheese. They further observed that Streptococcus paracitrovorus yielded cheese a higher flavor, which became slightly bitter as the cheese aged, and a smoother body and texture than normal cheese. These investigators also employed acid-liquefying cocci which yielded cheese a bitter flavor and a soft, pasty body and texture after two weeks of ripening.

Cultures of Streptococcus liquefaciens were used in addition to the normal lactic starter culture for making Cheddar cheese by Deane (22). He obtained a cheese which was equal to normal 18 week old cheese after six weeks of

ripening, but which had developed a bitter flavor after 12 weeks of ripening. Yates, et al. (84) inoculated S. liquefaciens into milk which was used for making Cheddar cheese. They obtained a cheese which had a bitter flavor and a weak body. These workers also observed that cheese made from milk inoculated with L. casei had an improved flavor after ten months of ripening. The absence of typical Cheddar cheese flavor in cheese made by employing pure cultures of Lactobacillus brevis and L. casei as the starter cultures was reported by Sharpe (60).

Sherwood (65) used a composite starter, containing Streptobacterium plantarum (L. plantarum) and S. cremoris, and low bacterial count milk to make Cheddar cheese. He found that the composite starter yielded a higher flavored cheese than did a pure starter culture of S. cremoris. However, Sherwood concluded that normal cheese milk would already contain enough lactobacilli to improve the cheese flavor. Sherwood (63) also used a mixed starter culture containing lactobacilli, and he obtained a cheese which had a strong fermented flavor. He isolated pure cultures of lactobacilli from the mixed culture and employed them in making cheese. Several of the pure cultures yielded cheese with the fermented flavor, while others improved the flavor of the resulting cheese.

Smith, et al. (72) did not observe that cultures of lactobacilli improved the flavor of Cheddar cheese. Lactobacilli were added to Cheddar cheese by Hunter (38). He incorporated the organisms into the liquid rennet extract, into the starter culture, or in a dry chalk-milk culture which was sprayed on the curd before hooping. Hunter observed that the chalk-milk culture gave the most increase in cheese flavor, followed by the starter culture, and that the inoculated rennet extract gave the least increase in cheese flavor.

Micrococci were found to improve the flavor of Cheddar cheese by Alford and Frazier (3). They added the micrococci cultures to cheese milk with the regular lactic starter culture. They ground the cheese after two to three weeks to hasten the ripening process. These investigators also added micrococci cells, recovered from carrot-liver broth by centrifugation, to freshly ground two to three week old Cheddar cheese. They reground the mixture, wrapped it in aluminum foil, and waxed it. The micrococci cells also improved the flavor of the resulting cheese. These workers could not determine the effect the micrococci had on the body of the cheese because it was ground. Harris and Hammer (32) found that several strains of micrococci and propionic bacteria improved Cheddar cheese flavor. Thirteen of 34 cultures of micrococci added to cheese milk with the lactic starter culture, improved the flavor of the resulting cheese according to Hunter (38). He

also found that seven of the micrococci cultures yielded a bitter flavor to cheese. Hunter also used cultures of propionic bacteria when making Cheddar cheese and noted that the resulting cheese had a slightly higher flavor than normal cheese. Hunter stated, "There remains the possibility that addition of one or more enzymes to the milk will be more practical than the use of a combination of bacteria."

Lane and Hammer (45) made Cheddar cheese from milk to which lipolytic enzymes had been added. They observed that pancreatin gave cheese a very disagreeable, rancid flavor. These workers also found that bovine mammary tissue, or water extracts of it, gave cheese a more desirable flavor after less than the normal length of ripening. Freeman and Dahle (30) added rennin, pepsin, or trypsin to milk before making cheese. They observed that rennin and pepsin increased proteolysis in the cheese, as shown by a weaker body, and that they both slightly increased the flavor of the cheese. These workers found that trypsin yielded cheese with a bitter flavor and a weak body.

Windlan and Kosikowski (83) reported obtaining a smooth bodied and desirable flavored cheese when adding a microbial enzyme system to the cheese. Kosikowski (42) later told of growing organisms in 60 gallon lots of whey and recovering the bacterial cells by centrifugation. These cells were then disrupted to liberate their enzymes. The enzymes were then

introduced into Cheddar cheese. The author was not able to find any reports of the results of these investigations in the literature.

Purification and Activity Determination of Bacterial Enzymes

Hugo (36) discussed several methods of releasing endocellular enzymes by disruption of bacterial cells, including grinding the cells with sand or ground glass and alternate freezing and thawing of the cells. Large numbers of S. lactis cells were harvested, by centrifugation from vitamin test medium, and disrupted with sonic vibration by Van der Zant and Nelson (79). These workers (78) also reported disrupting bacterial cells by grinding them with ground Pyrex glass and aluminium oxide (alumina). Van der Zant and Nelson (80) later described a procedure by which they were able to separate two proteolytic enzyme systems from a S. lactis cell-free extract by employing ammonium sulfate and 0.2 M phosphate buffer.

Koch and Ferrari (40) determined proteinase activity by the reduction in viscosity of a gelatin substrate. They measured the viscosity by timing the passage of a known quantity of gelatin through a funnel of specific size. Folin and Ciocalteau (27) presented a method for determination of the tyrosine-tryptophan content of a solution by the use of a phenol reagent.

Van der Zant (77) determined proteolytic activity on a two percent casein substrate by measuring the increase in the tyrosine-tryptophan content of the substrate. He used Folin and Ciocalteu's phenol reagent in a colorimetric determination described by Hull (37). Silverman and Kosikowski (68) reported a method for specific measurement of the free tyrosine in aged Cheddar cheese. In their procedure, an active bacterial decarboxylase converts free tyrosine into tyramine, which is measured colorimetrically using Folin and Ciocalteu's reagent. Silverman and Kosikowski stated that total free tyrosine was a relatively accurate measurement of protein degradation in ripened Cheddar cheese. Mogensen (50) observed that tyrosine-tryptophan measurement was suitable as a general indication of the amount of protein degradation that has occurred in Cheddar cheese.

Peterson, et al. (56) reported a method for determination of the proteinase content of Cheddar cheese. They measured the tyrosine-tryptophan content of a trichloroacetic acid filtrate of the cheese by employing Folin and Ciocalteu's reagent in a colorimetric determination. Northrup, et al. (51) described a casein digestion procedure for measuring proteolytic activity. They determined, by colorimetric measurement using Folin and Ciocalteu's reagent, the amount of casein digestion which had occurred during a specific period of time.

A lipase activity test for milk and cheese, based on the titration of butyric acid liberated from tributyrin, was reported by Peterson, et al. (53,54). Forster, et al. (28) described a colorimetric procedure for the measurement of lipolytic activity in milk. Barnett and Tawab (7) described a Soxlet extraction method removing volatile fatty acids from cheese. These fatty acids were then titrated to indicate the amount of fat hydrolysis which had taken place in the cheese. Frankel and Tarassuk (29) reported an ether extraction-titration procedure for determination of free fat acidity. Free fat acidity indicates the amount of fat hydrolysis which has occurred.

EXPERIMENTAL PROCEDURE

Proteolytic bacterial cultures, active at the pH and temperature of Cheddar cheese ripening, were isolated from raw milk. These bacterial cultures were screened by selecting those which produced a Cheddar cheese-like odor in milk. The selected bacterial cultures were used in the manufacture of Cheddar cheese and their subsequent effects upon the ripening of the cheese were observed.

Isolation, Propagation, and Screening of Proteolytic Cultures

Proteolytic bacterial cultures were isolated by streaking raw milk on skim milk-agar plates. The cultures were

propagated on nutrient agar slants and screened for production of Cheddar cheese-like odor in skimmilk and in whole milk. In order to have proteolytic cultures which would be active at the pH and temperature of cheese ripening, the isolations and screenings were carried out in media which had been adjusted to pH 5.4, and were incubated at 10°C.

Preparation of Media and Culture Milk. Skim Milk-Agar Plates. Reconstituted skim milk was prepared by dissolving ten g. of skim milk powder in 100 ml. of distilled water. Three percent agar was prepared separately, using distilled water. Both the skim milk and the agar were autoclaved for 15 minutes at 15 p.s.i. steam pressure and stored separately until the plates were prepared (within one week). Equal quantities of the autoclaved agar and skim milk were heated to approximately 60°C. for pouring the plates. Sterile ten percent lactic acid (approximately two ml./50 ml. agar) was added to the hot agar, in order that the resulting plates would have a pH of 5.4 ($\pm$ 0.05). Equal quantities of the agar and skim milk were thoroughly mixed and the plates poured to a depth of approximately one eighth of one inch.

Nutrient Agar Slants. Desiccated nutrient agar (Difco) was prepared according to directions and used to fill screw cap culture tubes approximately one third full. The culture tubes of medium were autoclaved for 15 minutes at 15 p.s.i. steam pressure and then allowed to solidify at an angle.

Culture Milk. Homogenized whole milk or ten percent reconstituted skim milk (as desired) was dispensed in 200 or 400 ml. quantities into flasks and autoclaved for 15 minutes at 15 p.s.i. steam pressure. After sterilization, the pH of the milk was adjusted to pH 5.4 ($\pm$ 0.05) with sterile ten percent lactic acid (approximately five ml/200 ml. milk).

Isolation and Propagation of Cultures. Raw milk samples were taken aseptically from the milk receiving line at a local dairy plant and from individual cows and mixed herd milk of the Kansas State College Dairy Herd. These samples were held in ice water, and streaked within two hours on skim milk-agar plates. These plates, prepared as described previously, were incubated at 10°C. ($\pm$ 1.0°) until clear zones, indicating proteolysis, had appeared, or for a period of 14 days. Isolated proteolytic colonies were restreaked on skim milk-agar plates and incubated as before. A proteolytic colony from each of the second plates was transferred to a nutrient agar slant for propagation. The nutrient agar slants, prepared as described before, were incubated at 10°C. and the cultures were transferred at least once every month.

Screening of Proteolytic Cultures. One loop (approximately 0.01 ml.) of bacterial cells from a nutrient agar slant of each culture, isolated as described above, was transferred

separately into 200 ml. of milk, prepared as previously outlined. Both reconstituted skim milk and whole milk were employed in the selection of cultures. The milk cultures were incubated at 10°C. ($\leq$ 1.0°) for a period of 30 days and were examined every other day for odor development. The bacterial cultures which produced a Cheddar cheese-like odor were selected for use in subsequent studies.

Determination of Proteolytic Culture Action in Whole Milk

Cultures of the proteolytic organisms, selected as described in the preceeding section, were tested in homogenized whole milk to determine if a correlation existed between the desirability of Cheddar cheese-like odor produced and the amount of protein degradation and/or the degree of fat hydrolysis that had occurred. The amount of protein degradation was indicated by the amount of tyrosine equivalent in an acid filtrate of the milk culture. The degree of fat hydrolysis was indicated by the free fat acidity of an ether extract of the milk culture.

Inoculation and Incubation of Milk Cultures. Flasks containing 400 ml. of homogenized whole milk, prepared as described previously, were each inoculated with one loop (0.01 ml. calibrated) of bacterial cells from a nutrient agar

slant of the selected proteolytic cultures. Tests showed that each 0.01 ml. loop of cells contained approximately four billion viable bacteria, as determined by a plate count procedure described in a subsequent section. The whole milk cultures, which had been adjusted to pH 5.4, were incubated at 10° C. (± 1.0°) for a period of 18 days. During this period, the cultures were tested, every other day, for the amounts of protein degradation and fat hydrolysis which had occurred, the desirability of Cheddar cheese-like odor, and the pH value of the milk culture.

Measurement of Degree of Fat Hydrolysis. A modification of the ether extraction-titration method of Frankel and Tarassuk (29) for determining the free fat acidity of milk, was employed as an indication of the degree of fat hydrolysis which had occurred in the milk cultures.

Reagents.

One percent alcoholic phenolphthalein - two g. of phenolphthalein and 100 ml. of 95 percent ethyl alcohol, diluted to 200 ml. with distilled water.

Extraction ether - a mixture containing 40 percent ethyl ether and 60 percent petroleum ether.

Neutralized alcohol - a mixture of 200 ml. of 95 percent ethyl alcohol and three ml. of one percent alcoholic phenolphthalein titrated to the phenolphthalein end-point with 0.05 N sodium ethylate.

Procedure. A 10 ml. aliquot of the milk culture to be tested was transferred by pipette to a 50 ml. centrifuge tube. Ten ml. of neutralized alcohol was added to the tube and the contents were thoroughly mixed by shaking the tube for 30 seconds. Fifteen ml. of extraction ether was added and the contents of the tube remixed for a second 30 seconds. The tube and its contents were centrifuged to separate the ether and milk-alcohol layers. Five ml. of the ether layer was immediately transferred into a 125 ml. flask containing 15 ml. of neutralized alcohol and this mixture was titrated to the phenolphthalein end-point with 0.05 N sodium ethylate. Free fat acidity was defined as the number of ml. of 1.0 N sodium ethylate needed to neutralized the total ether layer of milk containing 100 g. of butterfat. The fat content of the homogenized whole milk used in the cultures was determined by the Mojonnier ether extraction method.

Measurement of Protein Degradation. The method of Herriott (51) for the determination of the tyrosine-tryptophan content of a trichloroacetic acid filtrate was adapted to indicate the amount of protein degradation that had taken place in the milk cultures. The tyrosine-tryptophan content, hereafter referred to as the tyrosine equivalent, was determined from a tyrosine working curve prepared as described in a subsequent section.

Procedure. A ten ml. aliquot, of the milk culture to be tested, was placed in a 50 ml. beaker. Thirty ml. of five percent trichloroacetic acid was added to the milk and the mixture allowed to stand, at room temperature, for one hour. The precipitated milk-acid mixture was then filtered through Whatman No. 42 filter paper. The tyrosine equivalent of the trichloroacetic acid filtrate was determined by a procedure given in a later section of this manuscript.

Evaluation of Odors Produced. The whole milk cultures were scored by at least two experienced judges from the Department of Dairy Husbandry and the author for desirability of Cheddar cheese-like odor. A scoring range of one to ten was employed, with the highest score indicating the most desirable odor. The judges used whole point variations in scores to distinguish between degrees of desirability.

Determination of pH Value. The pH value of an aliquot sample of the culture being tested was determined with a Beckman Model H-2, line operated, pH meter. A glass indicating electrode and a saturated calomel referrence electrode were employed.

Statistical Analysis of Data. The data, analyzed by the Kansas State College Statistical Laboratory, consisted of an arithmetic average of the judges odor scores (Y), level of tyrosine equivalent (X_1), and the level of free fat acidity

(X_2) of the whole milk cultures. A multiple regression analysis was computed on each culture and since observation of the data indicated that a quadratic (single inflection) curve would fit the points better than a straight line, the following multiple regression equation was used.

$$\hat{Y}_{ij} = a + b_1X_1 + b_2X_1^2 + b_3X_2 + b_4X_2^2$$

Cheddar Cheese Making Procedure

Cheddar cheese, used for testing the effect of the selected bacterial cultures on ripening, was manufactured by the general procedure of Van Slyke and Price (81).

Pasteurization and Ripening of Cheese Milk. Unstandardized whole milk, pasteurized at 145° F. for 30 minutes and held overnight at 40° F., was employed in making Cheddar cheese. Fifteen or 50 gallons of milk (depending on the amount of curd desired) was placed in a 50 gallon, water jacketed, stainless steel vat and tempered to 88°F. A one and one-half percent inoculation of commercial lactic starter culture, which had been carried in sterile ten percent reconstituted skim milk, was added to the milk. The cheese milk was then allowed to ripen until a 0.01 percent increase in titratable acidity had occurred (25 to 30 minutes).

Addition of Cheese Color and Rennet, and Cutting the Curd. Cheese color was added at the rate of one and one-half ounces

per 1000 pounds of cheese milk. Commercial rennet extract was added to the cheese milk at the rate of four ounces per 1000 pounds of milk. The curd was cut in 25 to 30 minutes with three-eights inch wire knives, heated to 102° F., and held a period of one hour.

Draining the Whey, and Cheddaring, Milling, and Salting the Curd. At the end of the heating period, the whey was completely drained from the cheese vat and the cheese curd was piled approximately six inches deep along the sides of the vat. The cheese curd was Cheddared for approximately two and one-half hours. When the titratable acidity of the draining whey reached 0.42 percent, the cheese curd was milled and then salted at the rate of two and one-half pounds of salt per 1000 pounds of cheese milk.

Pressing, Waxing, and Ripening the Cheese. Approximately five pounds of the salted curd was placed in each rectangular cheese hoop and pressed for one hour. The cheese was removed from the hoop, wrapped in cheese cloth, and repressed overnight. The cheese was then placed in the ripening room for three days prior to waxing. After waxing, the cheese was stored in the cheese ripening room where the temperature was maintained at 50°F. ($\pm$ 2.0°).

Incorporation of the Bacterial Cultures in Cheese

Milk cultures of the proteolytic bacteria, selected as described in a preceding section, were incorporated into Cheddar cheese, during its manufacture, to determine their effects on the ripening process.

Viable Bacterial Counts. Viable bacterial counts of 24-hour and 72-hour old milk cultures, of the proteolytic organisms to be added to cheese, were determined to indicate approximately how many viable bacteria would be added to the cheese. Desiccated tryptone glucose yeast extract agar (Difco) was prepared according to directions and used for making the viable counts. The plates were incubated at 10°C. for five days and then the bacterial colonies present in the plate were counted.

Mixing Cultures with Cheese Curd. Separate trials, using 24-hour, 72-hour, and 25-day old whole milk cultures of each of the selected bacterial cultures, were run. Each trial entailed the mixing of 125 ml. of the desired milk culture with approximately five pounds of salted cheese curd just prior to "hooping", during the cheese manufacturing process previously described. It was found that larger quantities of milk culture would not be absorbed by the cheese curd. The cheese curd was manufactured from 50 gallon lots of whole milk.

Inoculation of Cheese Milk. Separate trials, employing 72-hour old skim milk cultures of each of the selected bacterial cultures, were run. Each trial consisted of the addition of one and two-thirds gallons of the desired milk culture to 15 gallons of cheese milk, just prior to the addition of the cheese color during the manufacturing process.

Injection of Cheese. It was proposed that an enzyme preparation could be injected into the cheese after it had been waxed, and that the preparation would diffuse throughout the cheese. To test this proposition, an aqueous solution of food coloring was injected into 60-day old cheese by means of a modified hypodermic needle. The needle was prepared by filing small holes in its side, for more even distribution of the liquid being injected. It was observed that very small quantities of the food coloring remained within the cheese and that the food coloring did not diffuse throughout the cheese as expected. From these observations, it was decided that injection of enzyme preparations into cheese would not be applicable to this study.

Evaluation of Cheddar Cheese Ripening

The effects that the different bacterial cultures, selected as described previously, had on the ripening of Cheddar cheese were shown by organoleptic evaluation of the cheese and by an

indication of the amount of cheese protein degradation which had occurred.

Organoleptic Evaluation of Cheese. The cheese, of the trials described in the preceding section, were scored every 30 days (± 3 days) by three experienced judges from the Department of Dairy Husbandry and the author. The cheese score card used in Collegiate Dairy Products Judging Contests was employed. This score card allocates 45 points to cheese flavor and 30 points to cheese body and texture. The scores of the four judges were averaged, to get the final cheese score.

Measurement of Cheese Protein Degradation. An acid filtrate of the cheese being tested was prepared by the method of Vakaleris and Price (76). In their procedure, a 10 g. representative sample of the cheese was placed in a Waring Blendor and thoroughly mixed with 40 ml. of 0.5 M sodium citrate and approximately 80 ml. of distilled water. The resulting cheese-citrate solution was further diluted to 200 ml. with distilled water. One hundred ml. of the diluted solution, ten ml. of 1.41 N hydrochloric acid, and enough distilled water to make a total quantity of 125 ml. were mixed and allowed to stand at room temperature for one hour. The precipitated solution was then filtered through Whatman No. 42 filter paper. The tyrosine equivalent of the acid filtrate, determined as described in a subsequent section, was used as an indication of the amount of cheese protein degradation which had taken place.

Statistical Analysis of Data. The data, analyzed by the Kansas State College Statistical Laboratory, consisted of the arithmetric average of the individual judges' scores of cheese flavor and cheese body and texture. An analysis of variance of each cheese trial, employing whole milk cultures, was determined. The means of the analysis of variance were compared by Ducan's (23) multiple range test. The last cheese trial, employing skim milk cultures, was not treated statistically because of its short duration.

Preliminary Attempts to Separate Crude Bacterial Enzymes

Some preliminary studies on the preparation of crude bacterial enzymes from whole milk cultures, which had been initially adjusted to pH 5.4 and were incubated at 10°C. were also performed. The determination of the fraction of a milk culture which contained enzyme activity was conducted. Enzyme preparations from cultures, which had been incubated for periods of either six days or 30 days, were made to determine if more enzyme activity existed in the older or younger culture. Enzyme activity was indicated by the measurement of the proteinase activity of the preparations.

Determination of Active Enzyme Fraction. A 30-day old whole milk bacterial culture was separated into a casein fraction, salt precipitated fraction, and a filtrate fraction

by the following procedure. The pH of a mixture of 400 ml. of the milk culture and 400 ml. of distilled water was lowered to pH 4.6 with ten percent lactic acid. The precipitated mixture was allowed to stand for 30 minutes, before the casein fraction was recovered by filtration through Whatman No. 41 filter paper, employing a Buechner type funnel and vacuum. The pH of the filtrate recovered was adjusted to pH 6.0 with 1.0 M ammonium hydroxide and then ammonium sulfate was added to 50 percent saturation (358g./liter). The salt precipitated fraction was recovered in the same manner as the casein fraction. The latter filtrate constituted the third fraction. Four hundred and fifty mg. of the casein fraction, 450 mg. of the salt precipitated fraction, and two ml. of the filtrate fraction were added separately to flasks containing 200 ml. of sterile whole milk, which had been adjusted to pH 5.4 as previously described. The flasks were incubated at 10°C. for a period of 30 days and were observed throughout the period for evidence of proteolysis and development of desirable Cheddar cheese-like odor.

Preparation of Crude Enzyme from Aged Milk Cultures. The casein fraction of a 30-day old whole milk bacterial culture was recovered. The casein fraction was stored at approximately -30°C., until it could be dried by the freeze-drying process

described in a following section. The proteinase activity of the dried preparation was determined as subsequently described. The aged culture was not alternately frozen and thawed as were the young cultures, because the bacterial cells were believed to be already autolized, thus having already released their endocellular enzymes.

Preparation of Crude Enzyme from Young Milk Cultures. A 100 percent whole milk bacterial culture and a 50 percent whole milk-50 percent distilled water bacterial culture were prepared and incubated for a period of six days, as previously described. At the end of the incubation period, the cultures were alternately frozen (-30°C.) and thawed (room temperature) five times to cause rupture of the bacterial cells, thus releasing their endocellular enzymes. Dried casein fractions of these cultures were then prepared by the same procedure used before. The proteinase activity of the dried preparations were determined as described later.

Freeze-Drying Procedure. Twenty ml. of the preparation to be dried, was frozen in a thin layer to the sides of a 200 ml. round bottom flask, by rotation of the flask and its contents in an alcohol-dry ice bath. These preparation flasks were stored overnight at -30°C. The freeze-drying apparatus consisted of a high vacuum pump and a freeze-out chamber in an

alcohol-dry ice bath. The freeze-out chamber had a side arm, on which the frozen preparation flasks were placed for drying. The entire system was placed under a high vacuum until the preparations were dry (approximately eight hours).

Determination of Proteinase Activity. The casein digestion method of Kunitz (Northrup, et al., 51) was adapted for use in this experiment. One percent buffered casein (Hammarsten quality) solutions were prepared by mixing the casein separately with buffers of the pH values 5.4, 5.6, and 6.6. Bacterial growth was inhibited by either three percent toluene or 40 percent sucrose concentration in the casein solutions. The completed solutions were autoclaved for 15 minutes at 15 p.s.i. steam pressure.

Procedure. Five ml. of the one percent buffered casein solution was placed in a sterile screw cap culture tube and ten mg. of the dried enzyme preparation, in one ml. of sterile distilled water, was added. This mixture was incubated at 10°C. for different lengths of time (depending on the activity of the preparation). At the end of the incubation period, 18 ml. of five percent trichloroacetic acid was added to the mixture and the precipitated solution was allowed to stand for 30 minutes. The acid filtrate was recovered by filtration through Whatman No. 42 filter paper. The tyrosine equivalent in the filtrate was determined by the procedure given below. The increase in tyrosine equivalent per day (24 hours) was used as an indication of the amount of proteinase activity present in the preparation.

Determination of Tyrosine Equivalent

A modification of the colorimetric method of Herriott (Northrup, et al., 51) for the measurement of the tyrosine-tryptophan content of an acid filtrate, was used in this study. The tyrosine-tryptophan content in an acid filtrate is referred to as its tyrosine equivalent in this manuscript. A standard working curve, using l-tyrosine, was prepared and used to determine the tyrosine equivalent in the acid filtrates from the amount of light that they absorbed. The tyrosine equivalent in an acid filtrate was used as an indication of protein degradation throughout the various phases of this study.

Development of Color. One ml. of the acid filtrate, in proper dilution to give a light transmission reading within the range of 20 percent to 95 percent transmission at 600 millimicrons wavelength of light, was placed in a 50 ml. flask. One ml. of a 0.0025 M copper sulfate solution and eight ml. of a 0.5 N sodium hydroxide solution were added to the flask and the contents of the flask were thoroughly mixed. Three ml. of dilute (one part to two parts distilled water) Folin and Ciocalteu's reagent was added dropwise to the flask with aggitation. The resulting color was observed to be unstable, therefore, light transmission readings were made exactly five minutes after the addition of the reagents.

Measurement of Light Transmission. A Bausch & Lomb Spectrophotometer, Model 20, was employed for the measurement of the percent light transmission through the colored solutions prepared as described above. Herriott (Northrup, et al., 51) recommended that light of 600 millimicrons wavelength be employed. The percent light transmission, at six different wavelengths of light, were taken for two dilutions of tyrosine (Table 1). It was observed that 625 millimicron light yielded slightly lower readings than 600 millimicron light. However, because some work already had been conducted at 600 millimicrons, this wavelength of light was employed in this study.

Table 1. Percent light transmission of two tyrosine solutions at six wavelengths of light.

Tyrosine dilution	Wavelength of Light (millicrons) 400	450	500	550	600	625
(mg./ml.)	(%L.T.)	(%L.T.)	(%L.T.)	(%L.T.)	(%L.T.)	(%L.T.)
0.050	62.0	61.0	51.5	44.5	39.0	38.0
0.100	85.0	79.0	71.0	64.0	60.0	56.0

Preparation of Tyrosine Working Curve. Distilled water solutions, containing 0.2 mg. of l-tyrosine per ml., were prepared in three trials. From these solutions, 15 successive dilutions were prepared in duplicate. Color was developed and percent light transmission of these dilutions was determined as

described above. The arithmetric average of the light transmission readings at each dilution (Table 2), was plotted on semi-logarithmic graph paper. A straight line, drawn by inspection through these points, served as the tyrosine working curve. (Fig. 1) for the subsequent determinations of tyrosine equivalent which have been previously described.

EXPERIMENTAL RESULTS

Proteolytic Bacterial Cultures Selected

Employing the methods described in Experimental Procedure, 30 proteolytic cultures were isolated from 128 raw milk samples. These cultures were purified and subsequently propagated on nutrient agar slants. Skim milk and whole milk cultures of the proteolytic bacteria were observed for the type of odor produced at 10°C. The skim milk cultures developed fruity, putrid, unclean, or yeasty odors. The whole milk cultures produced odors similar to those listed above, with the exception that four of the 30 proteolytic cultures yielded Cheddar cheese-like odors. These four bacterial cultures, No. I, No. II, No. III, and No. IV, were subsequently identified, by personnel of the Department of Bacteriology, as being members of the genus Lactobacillus. The four selected cultures were employed in the other phases of the study.

Table 2. Mean percent light transmission of 15 dilutions of tyrosine, used to prepare the tyrosine equivalent working curve.[1]

Tyrosine Dilution	Trial 1 Sample 1	Trial 1 Sample 2	Trial 2 Sample 1	Trial 2 Sample 2	Trial 3 Sample 1	Trial 3 Sample 2	Arithmetic mean
(mg./ml.)	(%L.T.)	(%L.T.)	(%L.T.)	(%L.T.)	(%L.T.)	(%L.T)	(%L.T.)
0.007	94.5	92.5	92.0	91.0	----	----	92.50
0.009	90.5	89.5	90.0	88.5	----	----	89.63
0.015	85.5	85.0	86.0	85.0	----	----	85.38
0.018	84.5	80.0	82.5	83.0	79.0	80.0	81.50
0.033	69.7	65.0	67.0	65.5	69.5	68.5	67.53
0.040	63.5	62.5	63.5	63.0	----	----	63.13
0.050	----	----	----	----	57.0	55.5	56.25
0.066	46.5	46.0	46.5	46.0	----	----	46.25
0.100	34.0	34.5	31.0	31.5	32.0	31.0	32.33
0.133	23.0	23.5	23.0	22.5	23.5	25.0	23.42
0.143	18.5	19.5	20.0	20.0	----	----	19.50
0.150	----	----	----	----	18.5	17.0	17.75
0.166	14.5	16.5	16.0	14.5	16.0	15.0	15.42
0.182	----	----	----	----	13.5	13.0	13.25
0.200	----	----	----	----	11.5	11.0	11.25

[1] Employing light of 600 millimicrons wavelength.

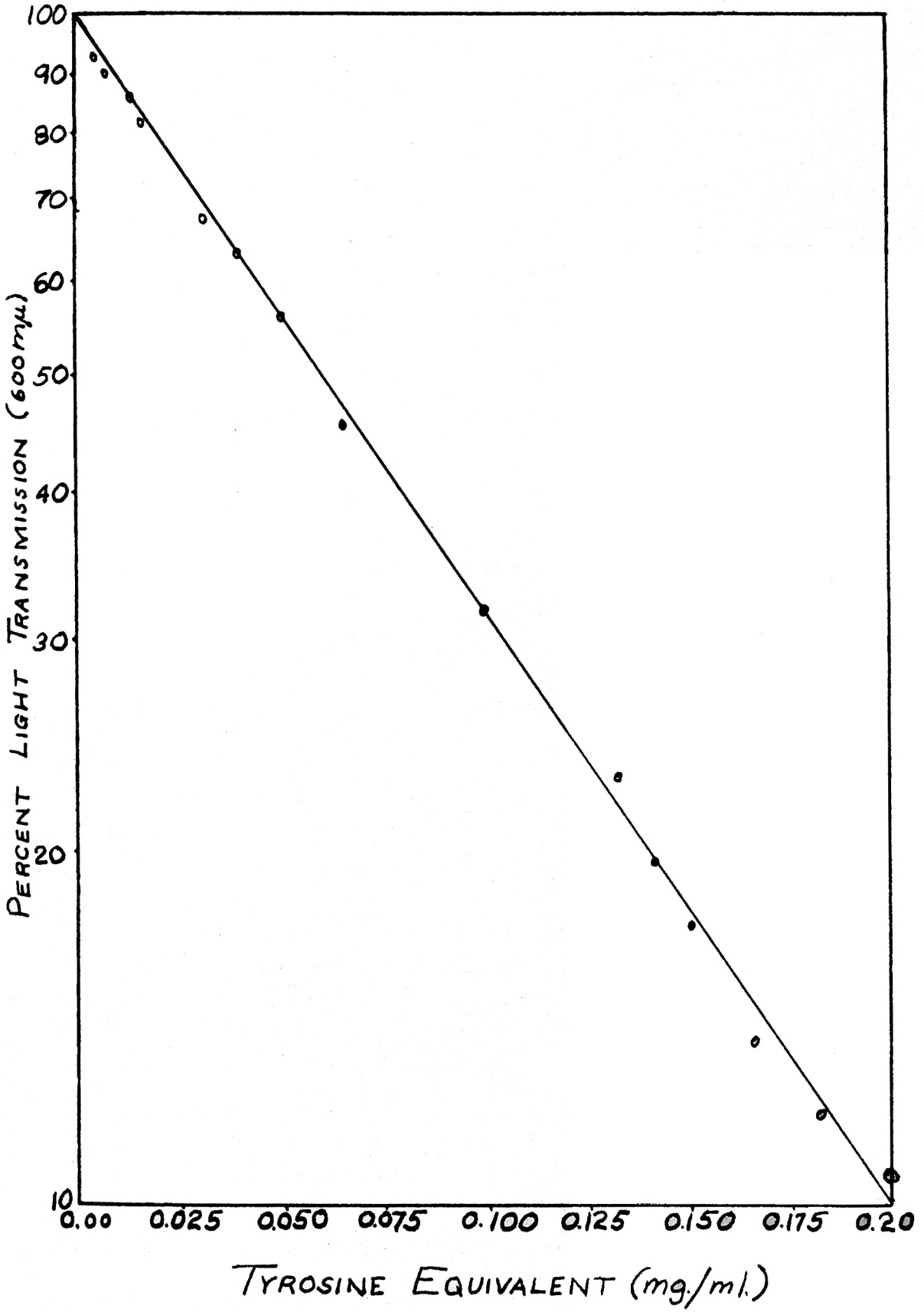

Fig. 1. Tyrosine equivalent working curve prepared from data in Table 2.

Action of Proteolytic Cultures in Whole Milk

The four proteolytic cultures were each studied in five separate trials employing homogenized whole milk, initially adjusted to pH 5.4, and an incubation temperature of 10°C. The individual cultures, within each trial, were tested initially and every second day for tyrosine equivalent, free fat acidity, pH value, and desirability of Cheddar cheese-like odor. Arithmetic means, of the factors determined in the five trials, were computed for each culture to make comparisons between the cultures simpler. A multiple regression analysis of the data obtained from each culture indicated whether protein degradation (tyrosine equivalent) and/or fat hydrolysis (free fat acidity) by the proteolytic culture were related to the desirability of the Cheddar cheese-like odor produced.

Proteolytic Culture No. I. Inspection of the data, collected in the five trials employing Culture No. I (Table 21, Appendix), showed that some variation existed between the trials. However, the computed means (Table 3) seemed to indicate the action of the culture to a reasonable degree. The mean levels of tyrosine equivalent, free fat acidity, and pH increased as the culture aged. Tyrosine equivalent reached an average level of 3.3 mg. per ml. of culture milk on the 18th day, while free fat acidity attained an average value of

approximately 28.0. The lower mean value of free fat acidity shown on the 18th day in Table 3 can be explained as the result of the lack of data on this day for one trial which had reached the highest level of free fat acidity. The maximum average desirability of odor score was 4.2 on the tenth day of incubation. The odor score decreased upon extended incubation.

Table 3. Arithmetic means of the factors determined in five trials testing the action of culture No. I in whole milk.[1]

Days of incubation	Mean pH of milk culture	Mean tyrosine equivalent (mg./ml.)	Mean free fat acidity	Mean desirability of odor
0	5.41	0.2684	5.0916	1.00
2	5.66	0.3360	4.3038	1.83
4	5.66	0.2930	4.4003	2.70
6	5.74	0.5692	5.0288	2.93
8	5.79	1.0624	6.3188	3.33
10	5.83	1.4670	7.9880	4.20
12	5.88	2.1900	13.5059	3.80
14	5.92	3.0256	20.5535	3.46
16	5.95	3.1590	27.4872	2.77
18	5.86	3.3330	25.3913	3.25

[1] Computed from data in Table 21, Appendix.

The multiple regression analysis of the data from the five trials testing Culture No. I indicated that 36 percent of the variability in desirability of odor could be explained by milk protein degradation (tyrosine equivalent) and milk fat hydrolysis

(free fat acidity). The estimations of the regression coefficients (Table 4) for tyrosine equivalent (b_1, b_2) are significantly different from zero, which indicates that protein degradation by this culture was related to desirability of odor. The negative value for b_2 implies that too much protein degradation was detrimental to desirability of odor. The regression coefficients for free fat acidity (b_3,b_4) were not statistically significant. The negative value of both b_3 and b_4 would seem to indicate that milk fat hydrolysis by this culture was detrimental to desirability of the odor produced. For optimum desirability of Cheddar cheese-like odor, the tyrosine equivalent level was calculated to be 3.0676, while the best positive level of free fat acidity would be zero.

Table 4. Regression coefficients of multiple regression analysis to relate tyrosine equivalent and/or free fat acidity with desirability of odor produced by cultures No. I and No. II.[1]

Determinations	Bacterial Cultures	
	No. I	No. II
Tyrosine equivalent		
linear (b_1)	1.6993**	1.9436*
quadratic (b_2)	-0.2770*	-0.3989 (.05<P<.10)
Free fat acidity		
linear (b_3)	-0.0154 (n.s.)	0.1444 (n.s.)
quadratic (b_4)	-0.0008 (n.s.)	-0.0046 (.05<P<.10)

[1] Computed from data in Tables 21 and 22, Appendix.

* P<.05.

** P<.01.

Proteolytic Culture No. II. The data from the five trials of Culture No. II (Table 22, Appendix) shows that some variation occurred between trials, but again, the computed means (Table 5) seemed to indicate the action of the culture. The mean levels of pH, tyrosine equivalent, and free fat acidity increased as the culture aged. The average level of tyrosine equivalent attained was 3.1 mg. per ml. of milk, which was slightly less than that attained by Culture No. I. Culture No. II did not reach as high a mean level of free fat acidity as did Culture No. I. The average desirability of odor produced by Culture No. II was the same as the previous culture, that is, 4.2 on the tenth day and decreasing on succeeding days. The low mean free fat acidity value and the high mean desirability of odor score on the 18th day was the result of a lack of data from one trial which previously had high free fat acidity values and low desirability of odor scores.

Table 5. Arithmetic means of the factors determined in five trials testing the action of Culture No. II in whole milk. [1]

Days of incubation	Mean pH of milk culture	Mean tyrosine equivalent (mg./ml.)	Mean free fat acidity	Mean desirability of odor
0	5.41	0.2684	5.0916	1.00
2	5.67	0.3467	4.5513	1.83
4	5.70	0.3418	3.9966	3.67
6	5.80	0.5628	5.3514	3.20
8	5.77	1.3260	6.2061	3.90
10	5.90	1.2510	8.4415	4.21
12	5.85	2.0220	12.1442	4.10
14	5.93	2.9125	15.9844	4.06
16	5.88	2.9656	22.4144	3.30
18	5.89	3.1185	18.7817	4.50

[1] Computed from Table 22, Appendix.

The multiple regression analysis of Culture No. II showed that 43 percent of the variability in desirability of odor, produced by the culture, could be explained by the combined effects of protein degradation and fat hydrolysis. The regression coefficients of the analysis of Culture No. II are reported in Table 4. The estimation of the linear regression coefficient for tyrosine equivalent (b_1) was the only coefficient significantly different from zero. However the quadratic coefficient for tyrosine equivalent (b_2) was near significance. The fact that b_2 was negative, means that an excess of protein degradation was detrimental to the desirability of the Cheddar cheese-like odor produced by this culture. Neither of the coefficients for free fat acidity (b_3,b_4) were significant, which indicates that fat hydrolysis was not important in the production of desirable odor by Culture No. II. The negative value for b_4 implies that too much fat hydrolysis by this culture was harmful to the desirability of the odor produced. Optimum desirability was calculated to occur when the levels of tyrosine equivalent and free fat acidity were 2.4360 and 1.5533, respectively.

Proteolytic Culture No. III. There was a great deal of variation in the data of the five trials employing Culture No. III (Table 23, Appendix). Two of the trials attained very high levels of free fat acidity. The lower mean level of free

fat acidity on the 18th day (Table 6) was the result of a lack of data for these two trials on that day. Culture No. III reached approximately the same mean level of tyrosine equivalent as did Culture No. I and No. II. The mean level of free fat acidity was 131.9 on the 16th day of incubation, which indicates that Culture No. III was much more lipolytic than either of the previous cultures. The desirability of odor reached by this culture was 7.5 on the tenth day, almost twice as high in desirability as the previous two cultures. The mean desirability of odor score decreased after the tenth day.

With Culture No. III, the multiple regression analysis showed that 46 percent of the variability in desirability of odor could be attributed to protein degradation (tyrosine equivalent) and fat hydrolysis (free fat acidity) by the culture. The regression coefficients (Table 7) for tyrosine equivalent (b_1,b_2) were nonsignificant statistically, which indicated that protein degradation by Culture No. III did not directly affect the desirability of the odor it produced. The linear coefficient for free fat acidity (b_3) was significant at the five percent level, meaning that fat hydrolysis was directly related to desirable odor production by this culture. The quadratic coefficient for free fat acidity (b_4) was near significance. The negative values for b_2 and b_4 indicated that both protein degradation and fat hydrolysis, respectively, were

detrimental to desirability of the Cheddar cheese-like odor in the later stages of growth. A tyrosine equivalent level of 1.9984 and a free fat acidity level of 150.1781 were calculated to be necessary for optimum desirability of odor in the milk.

Table 6. Arithmetic means of the factors determined in five trials testing the action of culture No. III in whole milk.[1]

Days of incubation	Mean pH of milk culture	Mean tyrosine equivalent	Mean free fat acidity	Mean desirability of odor
		(mg./ml.)		
0	5.41	0.2672	6.5000	1.00
2	5.78	0.3510	5.2057	2.25
4	5.83	0.3332	10.1950	4.63
6	5.95	1.2876	33.0268	5.46
8	5.82	1.5816	69.6756	6.20
10	5.81	2.3115	114.7031	7.46
12	5.75	2.4372	99.2827	5.40
14	5.83	2.5872	107.6077	5.47
16	5.71	3.0196	131.8825	5.20
18	5.85	3.1926	53.9020	4.83

[1] Computed from data in Table 23, Appendix.

Proteolytic Culture No. IV. Two of the five trials with Culture No. IV (Table 24, Appendix) reached higher levels of tyrosine equivalent and free fat acidity than did the other three trials. The lack of data for these two trials on the 18th day was the reason for the low mean tyrosine equivalent and low mean free fat acidity values shown on that day (Table 8). The mean levels of pH, tyrosine equivalent, and free fat acidity increased as the culture aged. Culture No. IV did not

reach as high a level of tyrosine equivalent as did the other three cultures, which indicates that it was not as proteolytic in nature. This culture attained a higher mean level of free fat acidity than did Cultures No. I and No. II, but it was not as lipolytic as Culture No. III. Again the highest mean odor score, 5.3, was attained on the tenth day of incubation and the mean score decreased on succeeding days.

Table 7. Regression coefficients of multiple regression analysis to relate tyrosine equivalent and/or free fat acidity with desirability of odor produced by cultures No. III and No. IV.[1]

Determinations	Bacterial Cultures	
	No. III	No. IV
Tyrosine equivalent		
linear (b_1)	1.9355 (n.s.)	2.6880*
quadratic (b_2)	-0.4843 (n.s.)	-0.6870 (n.s.)
Free fat acidity		
linear (b_3)	0.0439*	0.0339 (n.s.)
quadratic (b_4)	-0.0001 (.05<P<.10)	-0.0001 (n.s.)

[1] Computed from data in Tables 23 and 24, Appendix.
* P<.05.

The multiple regression analysis of Culture No. IV indicated that 54 percent of the variation in desirability of Cheddar cheese-like odor produced could be explained as the

result of protein degradation and fat hydrolysis by this culture. The regression coefficients estimations are reported in Table 7. The only coefficient significantly different from zero was the tyrosine equivalent linear coefficient (b_1). This indicates that protein degradation by this culture was directly related to production of desirable Cheddar cheese-like odor. The negative values of the quadratic coefficients for tyrosine equivalent (b_2) and free fat acidity (b_4) imply that beyond an optimum point, protein degradation and fat hydrolysis were detrimental to the desirability of the odor produced. The optimum levels of tyrosine equivalent and free fat acidity were calculated to be 1.9565 and 141.1292, respectively.

Table 8. Arithmetic means of the factors determined in five trials testing the action of culture No. IV in whole milk.[1]

Days of incubation	Mean pH of milk culture	Mean tyrosine equivalent (mg./ml.)	Mean free fat acidity	Mean desirability of odor
0	5.41	0.2672	6.5000	1.00
2	5.71	0.3165	4.9738	1.75
4	5.73	0.3256	6.0704	3.33
6	5.73	0.8780	12.0197	3.60
8	5.80	0.8088	24.5932	4.63
10	5.79	1.0125	43.4226	5.33
12	5.82	1.4140	51.7710	4.50
14	5.86	1.4540	63.2125	4.03
16	5.71	1.9614	77.0757	4.53
18	5.86	1.5030	53.8110	4.83

[1] Computed from data in Table 24, Appendix.

Effects of Proteolytic Cultures on Cheddar Cheese Ripening

Employing the methods outlined in Experimental Procedure, the effects of the four proteolytic bacterial cultures on the ripening of cheese were studied. Twenty-four-hour, 72-hour, and 25-day cultures of each organism in whole milk were added separately to freshly milled cheese curd (125 ml./5 pounds curd). The cheese were ripened for a period of 120 days. Viable bacterial counts indicated that approximately 20 million viable bacteria per ml. of 24-hour culture and 200 million viable bacteria per ml. of 72-hour culture were added to the cheese. Ten percent inoculations of 72-hour cultures of each organism in skim milk were added to separate vats of cheese milk. The cheese in this latter study had been ripened for only 60 days when the study was terminated. An analysis of variance of the data obtained from the studies employing whole milk cultures was computed. Data from the study employing skim milk cultures were not included because of the short duration of that study.

Twenty-Four-Hour Whole Milk Cultures Mixed with Cheese Curd. In two separate trials (Tables 9 and 10), the effects of each of the four selected organisms, in 24-hour whole milk cultures, on the ripening of Cheddar cheese were studied. The purpose of two identical trials was to determine if the effects

on ripening were reproducible. The tyrosine equivalent value increased as the cheese of both trials ripened, except for the determinations made at 90 days of ripening in Trial 1. It is presumed that the low readings recorded on this day are the result of an error in determination on that day.

An examination of the data in Table 9 and 10 revealed that the flavor and body and texture scores of the control cheese of each trial were reasonably close enough to infer good duplication between trials. The control cheese in Trial 1, after 120 days of ripening, had a flavor score of 39.00, flat and a body and texture score of 28.75, open and curdy, while the control cheese for Trial 2 scored 38.88, flat and 28.50, open. Culture No. I yielded a bitter flavor in cheese after 60 days of ripening in Trial 1 and after 90 days of ripening in Trial 2. After 120 days, the cheese containing this culture in Trial 1 scored 38.50, bitter and fermented and 28.50, open and weak and in Trial 2 scored 38.62, fermented and 28.87, open. Culture No. II produced a bitter flavor in cheese after 60 days of ripening in both trials. After 120 days of ripening, the cheese in Trial 1 had a flavor score of 38.50, bitter and a body and texture score of 28.50, open and pasty and the cheese in Trial 2 scored 38.12, unclean and 28.00, open and weak. Bacterial Culture No. III yielded a bitter flavor in cheese which was most intense after 90 days of ripening in Trial 1 and after 60 days in Trial 2. The

Table 9. Effect of 24-hour bacterial cultures on the ripening of cheddar cheese.[1]

Culture No.	Days of ripening	Tyrosine equivalent (mg./g.)	Flavor Average score	Flavor Criticism	Body & Texture Average score	Body & Texture Criticism
TRIAL 1						
Control	0	1.5900	----[2]	----	----	----
	30	3.8250	39.00	flat	28.00	open, curdy
	60	5.9125	38.50	flat, bitter, fermented	28.50	open, curdy
	90	3.9875	38.17	bitter, fermented	29.25	open
	120	6.6000	39.00	flat	28.75	open, mealy
I	0	1.5900	----	----	----	----
	30	4.1250	38.75	flat	28.00	open, curdy
	60	5.9125	38.83	flat	29.00	open
	90	4.1250	37.00	bitter	29.75	
	120	6.4625	38.50	bitter, fermented	28.50	open, weak
II	0	1.5900	----	----	----	----
	30	4.2750	39.00	flat	28.00	open, curdy
	60	6.3250	38.66	bitter	29.17	open
	90	4.5375	36.84	bitter	29.75	
	120	6.8750	38.50	bitter	28.50	open, pasty
III	0	1.5900	----	----	----	----
	30	3.9000	38.75	flat	28.00	open, curdy
	60	6.0500	38.66	bitter, fermented	29.17	open
	90	4.4000	36.84	bitter	29.25	open
	120	6.0500	38.70	bitter	28.75	open
IV	0	1.5900	----	----	----	----
	30	4.1250	39.25	flat	28.00	open, curdy
	60	6.8750	39.00	flat, bitter	29.00	open
	90	4.1250	36.17	bitter	29.50	
	120	6.4625	38.50	bitter	28.75	open

[1] 125 ml. of whole milk culture, adjusted to pH 5.4 and incubated at 10°C., was mixed with five pounds of cheese curd.

[2] Cheese was not scored initially.

Table 10. Effect of 24-hour bacterial cultures on the ripening of cheddar cheese.[1]

TRIAL 2

Culture No.	Days of ripening	Tyrosine equivalent (mg./g.)	Flavor: Average score	Flavor: Criticism	Body & Texture: Average score	Body & Texture: Criticism
Control	0	1.5900	----[2]	----	----	----
	30	4.1500	39.00	flat	28.00	open
	60	5.2250	38.50	flat	28.83	open, weak
	90	5.9125	37.88	bitter, fermented	28.50	open, pasty
	120	6.5360	38.88	flat	28.50	open
I	0	1.5900	----	----	----	----
	30	4.1500	39.50	flat	28.00	open, mealy
	60	5.9125	38.00	bitter, whey taint	28.83	open, weak, mealy
	90	6.4625	38.38	bitter, fermented	28.62	open, pasty
	120	6.1920	38.62	fermented	28.87	open
II	0	1.5900	----	----	----	----
	30	4.2000	39.00	flat	28.50	open
	60	5.5000	38.34	flat, bitter	28.17	open, mealy
	90	6.4625	37.62	bitter, whey taint	28.90	open
	120	6.5360	38.12	unclean	28.00	open, weak
III	0	1.5900	----	----	----	----
	30	4.1500	39.00	flat, bitter	28.75	open
	60	4.9500	37.00	bitter, fermented	28.84	open
	90	5.2250	38.00	bitter, fermented	28.12	open, weak
	120	6.0200	38.37	bitter, acid	28.62	open, weak
IV	0	1.5900	----	----	----	----
	30	4.3950	39.00	flat, bitter	28.75	open
	60	5.9125	38.83	bitter, fermented	28.83	open
	90	5.9125	38.75	bitter	28.38	open
	120	6.5360	38.62	fermented, whey taint	28.21	open, weak

[1] 125 ml. of whole milk culture, adjusted to pH 5.4 and incubated at 10°C., was mixed with five pounds of cheese curd.

[2] Cheese was not scored initially.

cheese in Trial 1 which contained this culture scored 38.70, bitter and 28.75, open after 120 days of ripening and the cheese in Trial 2 scored 38.37, bitter and acid and 28.62, open and weak after the same length of ripening. Culture No. IV produced a bitter flavor in cheese after 60 days in Trial 1 and after 30 days in Trial 2. This cheese, however, scored higher than the control cheese in both flavor and body and texture after 30 and 60 days of ripening in Trial 1 and after 30 days in Trial 2. After 120 days, the cheese containing Culture No. IV in Trial 1 scored 38.50, bitter and 28.75, open and in Trial 2 it scored 38.62, fermented and whey taint and 28.21, open and weak. Further examination of the data presented in Tables 9 and 10 revealed that the body and texture scores after 60 and 90 days of ripening, were consistantly higher in all the cheese in Trial 1 than in all the cheese in Trial 2.

Seventy-Two-Hour Whole Milk Cultures Mixed with Cheese Curd. Because the curd employed in this study was manufactured at the same time as the curd used in Trial 2 above, the control cheese for this study was the same as the one reported for Trial 2 above. Examination of the data in Table 11 showed that Culture No. I yielded a bitter flavor in cheese after 60 days of ripening and that after 120 days, the cheese had a flavor score of 37.50, bitter and fermented and a body and texture score of 28.50, open and weak. Culture No. II produced a bitter

Table 11. Effect of 72-hour bacterial cultures on the ripening of cheddar cheese.[1]

Culture No.	Days of ripening	Tyrosine equivalent (mg./g.)	Flavor: Average score	Flavor: Criticism	Body & Texture: Average score	Body & Texture: Criticism
Control	0	1.5900	----[2]	----	----	----
	30	4.1500	39.00	flat	28.00	open
	60	5.2250	38.50	flat	28.83	open, weak
	90	5.9125	37.88	bitter, fermented	28.50	open, pasty
	120	6.5360	38.88	flat	28.50	open
I	0	1.5900	----	----	----	----
	30	3.8250	39.50	flat	28.00	open, mealy
	60	5.0875	38.66	flat, bitter	29.00	open
	90	5.9125	38.00	bitter, fermented	28.62	open, pasty
	120	6.5360	37.50	bitter, fermented	28.50	open, weak
II	0	1.5900	----	----	----	----
	30	4.2000	37.84	flat, bitter	28.50	open
	60	5.9125	37.00	bitter, fermented	28.83	open
	90	5.9125	37.50	bitter, fermented, unclean	28.75	open
	120	6.8800	37.12	bitter, fermented	28.00	open, weak
III	0	1.5900	----	----	----	----
	30	4.2250	39.33	flat	28.00	open, curdy
	60	5.2250	37.67	bitter, fermented	28.83	open
	90	5.6375	38.00	bitter	28.50	open
	120	7.0520	38.12	bitter, whey taint	28.00	open, weak
IV	0	1.5900	----	----	----	----
	30	4.5500	39.33	flat, bitter	28.75	open
	60	5.9125	39.00	flat, bitter	29.34	open
	90	5.9125	38.75	bitter	28.38	open
	120	6.5360	38.75	whey taint	28.50	open

[1] 125 ml. of whole milk culture, adjusted to pH 5.4 and incubated at 10°C., was mixed with five pounds of cheese curd.

[2] Cheese was not scored initially.

flavor in cheese after 30 days of ripening and at 120 days the cheese scored 37.12, bitter and fermented and 28.00, open and weak. The cheese containing Culture No. III was bitter at 60 days and after 120 days of ripening it had a flavor score of 38.12, bitter and whey taint and a body and texture score of 28.00, open and weak. Culture No. IV yielded a slightly bitter flavor in cheese after 30 days of ripening. However, this cheese had a higher flavor score than the control cheese at 30, 60, and 90 days. The bitterness was not present in this cheese after 120 days of ripening, when the cheese scored 38.75, whey taint and 28.50, open. The data in Table 11 reveals that tyrosine equivalent increased as the cheese aged.

Twenty-Five-Day Whole Milk Cultures Mixed with Cheese Curd. The data in Table 12 show that the control cheese for this study, after 120 days of ripening, had developed a bitter flavor and it scored 38.33, flat and bitter and 28.38, curdy and mealy. Bacterial Culture No. I yielded a bitter flavor in cheese after 30 days of ripening. At 120 days, this cheese had a flavor score of 37.25, bitter and a body and texture score of 28.88, open. The cheese containing Culture No. II developed a bitter flavor after 30 days of ripening. At 120 days, this cheese scored 37.38, bitter and 28.50, open. Culture No. III produced a bitter flavor in cheese after 30 days of ripening and after

Table 12. Effect of 25-day bacterial cultures on the ripening of cheddar cheese.[1]

Culture No.	Days of ripening	Tyrosine equivalent (mg./g.)	Flavor: Average score	Flavor: Criticism	Body & Texture: Average score	Body & Texture: Criticism
Control	0	1.3500	----[2]	----	----	----
	30	4.4500	28.50	flat	28.50	corky
	60	5.0870	39.00	flat	28.50	curdy
	90	5.2250	37.00	acid, unclean	28.25	open, mealy
	120	6.4625	38.38	flat, bitter	28.38	curdy, mealy
I	0	1.3500	----	----	----	----
	30	5.4000	37.30	bitter	28.00	corky
	60	5.5000	37.50	bitter	28.75	curdy
	90	7.0125	35.00	bitter	29.00	open
	120	7.8375	37.25	bitter	28.88	open
II	0	1.3500	----	----	----	----
	30	4.3000	38.00	bitter	28.00	corky
	60	6.8750	38.50	flat	28.50	curdy
	90	4.5375	35.00	bitter	29.00	open
	120	6.8750	37.38	bitter	28.50	open
III	0	1.3500	----	----	----	----
	30	4.6000	37.00	bitter	28.00	corky
	60	6.8750	37.50	bitter, rancid	28.85	curdy
	90	7.1500	36.00	bitter	28.75	curdy
	120	6.8750	37.00	bitter, unclean	28.75	open
IV	0	1.3500	----	----	----	----
	30	4.7000	38.70	flat	28.50	corky
	60	5.7750	37.50	bitter, rancid	28.75	curdy
	90	6.1875	36.75	bitter	28.75	curdy
	120	7.1500	37.00	bitter, rancid	29.00	open

[1] 125 ml. of whole milk culture, adjusted to pH 5.4 and incubated at 10°C., was mixed with five pounds of cheese curd.

[2] Cheese was not scored initially.

120 days it scored 37.00, bitter and unclean and 28.75, open. The cheese containing Culture No. IV scored higher than the control cheese after 30 days of ripening. This cheese developed a bitter flavor after 60 days of ripening and after 120 days scored 37.00, bitter and rancid and 29.00, open. The tyrosine equivalent values increased as the cheese aged in this study.

Seventy-Two-Hour Skim Milk Cultures Added to Cheese Milk. Because the skim milk cultures were added separately to the individual vats of cheese milk, it was not possible to have a control cheese for this study. Examination of the data assembled in Table 13 indicated that the cheese containing Culture No. I did not develop a bitter flavor in 60 days of ripening. Culture No. II yielded a bitter flavor in cheese after 60 days of ripening. The cheese containing Culture No. III had a strong rancid and unclean flavor and a weak body. This cheese was unpalatable. Culture No. IV also produced strong rancid and unclean flavors in cheese after 60 days of ripening.

Statistical Analysis of Cheese. Compiled in Table 14 are the data computed in analyses of variance of the cheese studies employing whole milk bacterial cultures. Examination of the analysis of variance, with respect to flavor scores, indicates that culture, age of culture, days of ripening and the interaction between age of culture and days of ripening

Table 13. Effect of 72-hour bacterial cultures added to the cheese milk on the ripening of cheddar cheese.[1]

Culture No.	Days of ripening	Tyrosine equivalent (mg./g.)	Flavor Average score	Flavor Criticism	Body & Texture Average score	Body & Texture Criticism
I	0	1.2375	----[2]	----	----	----
	30	4.0500	28.75	flat	28.63	pasty
	60	6.3250	38.66	acid	28.16	pasty, weak
II	0	1.1000	----	----	----	----
	30	4.8000	38.13	acid	28.75	pasty
	60	5.9125	37.00	acid, bitter	28.00	curdy, short
III	0	0.9625	----	----	----	----
	30	4.8750	35.35	rancid, unclean	28.75	pasty
	60	5.2250	34.33	rancid, unclean	27.50	weak, open
IV	0	1.0175	----	----	----	----
	30	4.8750	37.00	unclean	28.00	pasty
	60	5.2250	36.33	rancid, unclean	27.33	pasty, weak

[1] 1.6 gallons of skim milk culture, adjusted to pH 5.4 and incubated at 10°C., was added to 15 gallons of cheese milk.

[2] Cheese was not scored initially.

were all significant sources of variation at the one-tenth of one percent level. The interaction between age of culture and culture was a significant source of variation at the five percent level. The nonsignificance of the interaction between culture and days of ripening indicated that each culture does not have a separate, optimum length of ripening, with respect to flavor development.

Table 14. Analyses of variance of cheese studies employing whole milk cultures.[1]

Source of variation		Degree of freedom	Flavor Mean square	Flavor F	Body & Texture Mean square	Body & Texture F
Age of culture	(A)	3	5.7190	25.89***	0.3164	5.70**
Culture	(C)	4	1.5972	7.24***	0.1163	2.10 (n.s.)
Days of ripening	(D)	3	7.4178	33.63***	1.7213	31.01***
A x C		12	0.5385	2.44*	0.0344	0.62 (n.s.)
A x D		9	1.0523	4.77***	0.13982	7.17***
C x D		12	0.1419	0.68(n.s.)	0.1333	2.40*
Remainder		36	0.2206		0.0555	

[1] Computed from data in Tables 9 to 12, Appendix.

* $P < .05$

** $P < .01$

*** $P < .001$

The analysis of variance of body and texture in the cheese trials is reported in Table 14. These data show that days of ripening and the interaction between age of culture and days of ripening were a significant source of variation at the one-tenth of one percent level, while age of culture alone was significant at the one percent level. The interaction between culture and days of ripening was significant at the five percent level. The nonsignificance of culture as a source of

variation indicates that none of the four cultures were better than the rest in respect to their effect on cheese body and texture score. The age of culture and culture interaction was nonsignificant, which implies that none of the four cultures, at any of the specific ages, 24-hour, 72-hour, or 25-day, produced a more desirable body and texture than any of the others.

The significant sources of variation in the cheese, with respect to cheese flavor, were compared by a Multiple Range Test devised by Duncan (23). The results of these comparisons are reported in Table 15.

Table 15. Individual comparisons within significant sources of variation of cheese flavor by Duncan's multiple range test.

Source of variation :	Comparisons				
Age of culture (A)					
Ranked A	25-day	72-hour	24-hour (Trial 1)	24-hour (Trial 2)	
Ranked means[1]	37.313	38.317	38.334	38.471	[2]
Culture (C)					
Ranked C	No. II	No. III	No. I	No.IV	Control
Ranked means	37.776	37.871	38.018	38.369	38.504
Days of ripening (D)					
Ranked D	90 days	120 days	60 days	30 days	
Ranked means	37.277	38.160	38.258	38.738	

[1] Mean flavor scores.

[2] Underscored indicates means were not significantly different.

The data in Table 15 indicate that cultures which were 24 hours and 72 hours old when employed in cheese making were equally good and were superior to 25-day old cultures with respect to cheese flavor development. The ranking of the cultures with respect to mean flavor score, indicates that Culture No. IV was the best, followed by Cultures No. I, No. III, and No. II, respectively. The mean flavor score for the control cheese was higher than any of the mean flavor scores for the cheese containing the cultures. However, Duncan's Multiple Range Test indicated that the mean flavor score produced by Culture No. IV was not significantly different than that of the control. The data in Table 15 show that the cheese had the highest mean flavor at 30 days and the lowest mean flavor after 90 days of ripening.

The data compiled in Table 16 are the results of comparisons of the significant sources of variation with respect to cheese body and texture scores. Duncan's Multiple Range Test was again employed to make the comparisons.

Examination of the data in Table 16 shows that in the ranking of the age of culture, Trials 1 and 2 of the 24 hour cultures formed the two extremes of the ranking. Because of this lack of repeatability, it was impossible to determine which culture age would be the best with respect to body and texture. The comparisons of the days of ripening showed that 60 or 90 days of ripening were the best, and that 120 days was

significantly better than 30 days of ripening, with respect to cheese body and texture.

Table 16. Individual comparisons within significant sources of variation of cheese body and texture score by Duncan's multiple range test.

Source of variation :	Comparisons			
Age of culture (A)				
Ranked A	24-hour (Trial 2)	72-hour	25-day	24-hour (Trial 1)
Ranked means[1]	28.511	28.517	28.581 [2]	28.780
Days of ripening (D)				
Ranked D	30 days	120 days	60 days	90 days
Ranked means	28.213	28.523	28.826	28.826

[1] Mean body and texture scores.

[2] Underscored indicates means were not significantly different.

Preliminary Attempts to Separate Crude Enzyme Preparations

Fraction of Whole Milk Culture Containing Active Enzyme. Employing the methods described in Experimental Procedures, approximately 80 grams of casein fraction, 450 mg. of 50 percent saturated salt fraction, and the filtrate fraction were recovered from 400 ml. of 30-day whole milk bacterial Culture No. I. Specific quantities of each fraction were added

separately to flasks of sterile whole milk. The odor present in the milk and the tyrosine equivalent contained in an acid filtrate of the milk after 30 days of incubation at 10°C. were determined to indicate enzyme activity. It was found (Table 17) that the casein fraction produced a Cheddar cheese-like odor and also had the highest tyrosine equivalent, both of which indicated that the bacterial enzymes were in this fraction. Hereafter the casein fraction from a bacterial milk culture will be referred to as crude enzyme.

Table 17. Enzyme activity of three fractions of 30 day whole milk culture No. I.[1]

Milk fraction added	Amount fraction added	Tyrosine equivalent at end of 30 days (mg./ml.)	Odor present
Control	none	0.480	cooked
Casein	450 mg.	9.000	Cheddar cheese-like
Salt precipitated	450 mg.	3.200	cooked
Filtrate	2 ml.	0.340	cooked

[1] Activity in whole milk, initially adjusted to pH 5.4 and incubated at 10°C. for 30 days.

Activity of Crude Enzyme Preparations from Aged and Young Cultures. The methods outlined in Experimental Procedure were used to determine the activity of crude enzyme prepared from

30 day whole milk cultures No. II and No. IV and from six day cultures of organism No. II in 100 percent whole milk and in 50 percent whole milk. The increase in tyrosine equivalent per 24 hours during the activity test period was used for comparisons of the crude enzymes. Table 18 indicates that a 11 day test period was better than an 18 hour test period because the larger amounts of tyrosine equivalent attained could be more accurately determined. The data in Table 19 show that the crude enzymes from Cultures No. II and No. IV were more active at pH 6.6 than at pH 5.6. Tables 18 and 19 indicate that these crude enzymes were also more active at pH 5.6 than at pH 5.4. Table 20 shows that more proteolytic enzyme activity per unit volume was obtained in a crude enzyme preparation from 50 percent whole milk bacterial culture than from 100 percent whole milk bacterial culture. The data in Table 19 and 20 indicate that more enzyme activity per unit volume was recovered from aged cultures than from young cultures.

Table 18. Proteolytic enzyme activity at pH 5.4 of crude enzyme preparations from 30 day whole milk cultures No. II and No. IV. [1]

Culture No.	Amount of crude enzyme (mg.)	Length of test period	Tyrosine equivalent Initial	Final	Increase	Increase/day
			(mg./mg. crude enzyme)			
II	10	18 hours	0.0120	0.0156	0.0036	0.0048
IV	10	18 hours	0.0115	0.0168	0.0053	0.0071
II	10	11 days	0.0120	0.0470	0.0350	0.0031
IV	10	11 days	0.0110	0.0220	0.0110	0.0010

[1] Activity on two percent casein solution, which contained three percent toluene and was buffered at pH 5.4.

Table 19. Proteolytic enzyme activity at pH 6.6 and 5.6 of crude enzyme preparations from 30 day whole milk culture No. II and No. IV.[1]

Culture No.	Amount of crude enzyme	pH of casein solution	Tyrosine equivalent			
			Initial	Final	Increase	Increase/day
	(mg.)		(mg./mg. crude enzyme)			
II	10	6.6	0.0120	0.0830	0.0710	0.0064
IV	10	6.6	0.0110	0.0440	0.0330	0.0030
II	10	5.6	0.0120	0.0670	0.0550	0.0050
IV	10	5.6	0.0110	0.0370	0.0260	0.0023

[1] Activity for a 11 day period on a two percent casein solution, which contained 40 percent sucrose and was buffered at pH 5.6 or 6.6

Table 20. Proteolytic enzyme activity at pH 6.6 and 5.6 of crude enzyme preparations from six day cultures of bacteria No. II in 100 percent and 50 percent whole milk.[1]

Culture medium	Amount of crude enzyme	pH of casein solution	Tyrosine equivalent			
			Initial	Final	Increase	Increase/day
	(mg.)		(mg./mg. crude enzyme)			
100%milk	10	6.6	0.0430	0.0580	0.0150	0.0015
100%milk	10	5.6	0.0430	0.0480	0.0050	0.0005
50%milk	10	6.6	0.0260	0.0580	0.0320	0.0032
50%milk	10	5.6	0.0260	0.0360	0.0100	0.0010

[1] Activity for a 10 day period on a two percent casein solution, which contained 40 percent sucrose and was buffered at pH 5.6 or 6.6.

DISCUSSION

At the XIIIth International Dairy Congress, 1953, Pette (58) stated "...research work on cheese ripening during the last five years has been mainly carried out in two directions, viz.:

1. analysis of flavour substances, especially decomposition products of casein and fat;
2. investigations on the influence of different kinds of bacteria and their enzymes on cheese flavor."

The study reported in this manuscript falls into Pette's second classification. Many investigations have been directed towards this phase of studying Cheddar cheese ripening, but to the author's knowledge, none of these investigations used an initial approach to the problem similar to the isolation and selection of the bacteria employed in this study.

Many investigators have pointed out the fact that raw milk Cheddar cheese normally has a higher, more desirable flavor than pasteurized milk Cheddar cheese of the same age. Because of the large number of micrococci present in raw milk, Alford and Frazier (3) studied the effects of these organisms on Cheddar cheese flavor development. They observed that micrococci improved cheese flavor. Other investigators, such as Dahlberg and Kosikowski (17), have used specific strains of organisms in attempts to improve the flavor of cheese. Dahlberg

and Kosikowski employed S. faecalis and found that this organism improved the flavor of cheese only when very low bacterial count milk was employed.

Because of the accepted fact that raw milk cheese is superior to pasteurized milk cheese, it was decided to isolate organisms from raw milk for use in attempts to hasten the ripening of Cheddar cheese. It was further decided to limit this study to proteolytic organisms which are active at the pH and temperature of ripening Cheddar cheese. To accomplish this, the isolation and selection of the bacteria were made at pH 5.4 and at 10°C. Albrect and Jaynes (1) found that inorganic acids inactivated milk lipase, while lactic acid did not. Therefore, lactic acid was used exclusively for lowering the pH of the bacterial medium in this study.

In the selection of the bacteria, it was found that Cheddar cheese-like odors were produced in whole milk and not in skim milk. This supports the statement of Babel and Hammer (4) that milk fat was necessary for the production of Cheddar cheese flavor.

In the statistical analysis of the data collected in the study of the action of the selected bacteria in whole milk, it was found that 36 to 54 percent of the variation in the desirability of the Cheddar cheese-like odor could be explained

by protein degradation (tyrosine equivalent) and fat hydrolysis (free fat acidity). Several explanations of this seemingly low correlation are offered. First, the method of determining the degree of free fat acidity may have had inherent errors. Secondly, the measurement of free fatty acids may not have been a true measurement of fat hydrolysis, as free fatty acids could and probably did arise from the deamination of amino acids. The third explanation offered is that protein degradation and fat hydrolysis, per se, may not be correlated with Cheddar cheese-like odor production. The odor was undoubtedly the result of decomposition of the milk by the bacteria and their enzymes. However, it may be that the cheesy odor producing compound(s) were the product(s) of subsequent reactions involving the amino acids and fatty acids from the decomposition of milk. Keeney and Day (39) and Jackson (59) both reported obtaining cheesy flavored aldehydes from methionine.

The statistical analysis of the bacterial action in whole milk also showed that protein degradation and fat hydrolysis by each culture could proceed too far with respect to desirability of odor. This finding also supports the third explanation of the seemingly low correlation given above. That is, accumulations of the protein and fat decomposition end-products were harmful to the desirability of the odor produced. In all four cultures, the highest mean desirability of odor was

attained on the tenth day of ripening, which also indicates that accumulation of end-products had an adverse effect. The fact that the highest mean odor scores were on the tenth day of incubation also suggests that the cheesy odor in milk was the result of either bacterial metabolism or exoenzymes released by the bacteria. This seems to be contrary to the findings of Peterson, et al. (57), who believed that Cheddar cheese ripening (including flavor production) was largely the result of endoenzymes released upon bacterial autolysis. However, it must be remembered that the milk cultures did not have the same environmental conditions found in cheese, such as they lacked the salt concentration and anaerobic conditions.

Bacterial Culture No. IV appeared to be the most promising culture as far as desirable effects on Cheddar cheese ripening were concerned. The mean cheese flavor score (of the entire 120 day ripening period) for this culture was higher than that of any of the other three cultures. However, it was the same as the mean flavor score for the control cheese. An examination of the individual cheese scores showed that all of the cheese containing Culture No. IV scored higher than the control cheese in both flavor and body and texture after 30 days of ripening. The cheese containing the 24-hour and 72-hour cultures of No. IV also scored higher after 60 days of ripening. The judges remarked that these cheese were more fully ripened than the control cheese. It might be well to

point out one fallacy in the method employed for scoring the cheese flavors. A young cheese which has a clean, but flat flavor rates a relatively high score, as does a well ripened, full flavored cheese. It is the opinion of the author that scoring of the cheese by the conventional flavor standards did not fully indicate whether or not one cheese was more fully ripened than another. Bacterial Culture No. IV yielded a bitter and/or fermented flavor in cheese in the latter stages of ripening. It is of interest to note that this culture differed from the other cultures in its action in whole milk. Culture No. IV was more lipolytic than Cultures No. I and No. II and it was the least proteolytic of any of the cultures. The other three cultures predominantly yielded a bitter flavor in cheese. This does not, however, rule out the possibility that enzyme preparations from these organisms would have a desirable effect on the ripening of Cheddar cheese.

From the preliminary preparation of crude enzyme, the following conclusion can be drawn. The bacterial enzyme systems were separated from the milk cultures with the casein as it was precipitated at its isoelectric point, pH 4.6. The use of such crude enzyme preparations in the manufacture of Cheddar cheese remains to be studied. Alford and Frazier (3) recovered bacterial cells from liver-carrot broth and added them to freshly ground Cheddar cheese. It may be that the

bacteria employed in this study could be grown in a synthetic medium, and bacterial cells subsequently recovered. These cells could then be incorporated into cheese and their subsequent effect on the ripening of cheese studied. The cells could also be ruptured to release their enzyme systems and these enzyme systems separated for use in Cheddar cheese.

Since fat degradation seems to be essential for the production of desirable cheese flavor, the isolation of bacteria from raw milk on the basis of lipolytic activity would be another approach for the continuation of this study. The results of this study are promising enough to necessitate further investigations concerning the possible use of added bacterial enzymes for accelerating the ripening of Cheddar cheese.

SUMMARY AND CONCLUSIONS

The study reported in this manuscript was the preliminary work of a project involving the use of added bacterial enzyme systems as a means of accelerating Cheddar cheese ripening. One hundred and twenty eight raw milk samples were obtained and from these samples, 30 proteolytic bacterial cultures were isolated under pH and temperature conditions similar to those found in ripening Cheddar cheese. The proteolytic cultures were screened by observing the odors they produced in milk. The four cultures employed in the subsequent

studies were selected because they produced a Cheddar cheese-like odor in whole milk. These cultures were subsequently identified as members of the genus Lactobacillus.

The action of the selected cultures in whole milk was studied. It was determined statistically that 36 to 54 percent of the variation in the desirability of the Cheddar-cheese-like odor produced by the bacteria could be explained by protein degradation (tyrosine equivalent) and fat hydrolysis (free fat acidity). It was also observed that too much protein degradation and too much fat hydrolysis were detrimental to the desirability of the odor produced. The highest mean odor scores were attained by all the cultures on the tenth day of incubation. This suggested that the odor was the result of bacterial metabolism or exoenzymes from the bacteria.

The effects of the four cultures on the ripening of Cheddar cheese were studied. Cultures No. I, No. II, and No. III yielded a flavor and body and texture in cheese which scored lower than that of the control cheese. The predominant off-flavor defects caused by these cultures were bitter and fermented. Culture No. IV yielded a flavor and body and texture in cheese which scored higher than the control cheese at 30 and 60 days of ripening. This cheese scored slightly less than the control cheese after 90 and 120 days of ripening. An analysis of variance of the cheese studies indicated that culture, age of culture, days of ripening, interaction between

culture and age of culture, and the interaction between days of ripening and age of culture were significant sources of variation with respect to cheese flavor. Another analysis of variance showed that age of culture, days of ripening, interaction between age of culture and days of ripening, and the interaction between culture and days of ripening were significant sources of variation with respect to cheese body and texture.

Preliminary work with the preparation of crude enzymes from whole milk cultures of the bacteria indicated that the enzyme systems were separated from the cultures with the precipitation of the casein at its isoelectric point.

Several suggestions for the continuation of the project were made, including the isolation of lipolytic bacteria from raw milk and the preparation of enzymes from the cultures employed in this study.

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APPENDIX

Table 21. Action of proteolytic culture No. I in whole milk.

Trial	Days of incubation	pH of milk culture	Tyrosine equivalent (mg./ml.)	Free fat acidity	Desirability of odor
1.	0	5.40	0.2920	4.4020	1.00
	2	5.65	0.3020	3.4842	2.00
	4	5.80	0.2440	4.5845	3.00
	6	5.85	0.2780	7.1518	3.00
	8	5.65	0.5280	4.0348	4.00
	10	5.90	1.0320	10.0870	5.50
	12	6.00	2.0400	20.7242	6.00
	14	5.80	2.5440	24.3922	5.00
	16	5.90	3.2780	29.5274	3.00
	18	6.00	3.6960	23.1084	3.00
2.	0	5.40	0.2560	10.2956	1.00
	2	5.85	0.4040	5.8832	1.50
	4	----[1]	0.3320	8.4571	4.00
	6	5.75	0.6960	8.0894	3.00
	8	6.10	1.2960	16.1788	3.00
	10	6.00	2.0160	12.8695	3.00
	12	5.85	2.4120	17.6496	2.00
	14	6.10	3.1680	38.6085	1.66
	16	6.15	3.2780	51.8457	1.50
	18	----	-----	-----	----
3.	0	5.45	0.2700	4.2528	1.00
	2	5.50	0.3020	3.5440	2.00
	4	5.70	0.2780	2.8352	3.50
	6	5.80	0.7800	3.0124	2.66
	8	5.70	1.2000	3.7212	4.00
	10	----	----	----	----
	12	5.90	2.7360	6.3792	5.00
	14	5.80	5.3020	14.8848	4.00
	16	5.90	3.9160	24.0992	3.33
	18	6.00	3.6080	24.0992	3.00
4.	0	5.40	0.2620	3.2538	1.00
	2	----	----	----	----
	4	5.60	0.3320	2.8710	1.50
	6	5.70	0.5760	3.0624	3.00
	8	5.80	1.4880	3.8280	2.66
	10	5.80	1.5720	5.1678	4.00
	12	5.85	1.8260	11.8668	3.00
	14	5.90	2.0680	14.3550	3.00
	16	5.95	2.6400	16.8432	3.00
	18	5.75	3.1460	26.4132	3.00

Table 21 (concl.)

Trial	Days of incubation	pH of milk culture	Tyrosine equivalent (mg./ml.)	Free fat acidity	Desirability of odor
5.	0	5.40	0.2620	3.2538	1.00
	2	----	----	----	----
	4	5.55	0.3000	3.2538	1.50
	6	5.60	0.5160	3.8280	3.00
	8	5.70	1.2000	3.8280	3.00
	10	5.60	1.2480	3.8280	4.33
	12	5.80	1.9360	10.9098	3.00
	14	6.00	2.0460	10.5270	3.66
	16	5.85	2.6840	15.1206	3.00
	18	5.70	2.8820	27.9444	4.00

[1] Data not available.

Table 22. Action of proteolytic culture No. II in whole milk.

Trial	Days of incubation	pH of milk culture	Tyrosine equivalent (mg./ml.)	Free fat acidity	Desirability of odor
1.	0	5.40	0.2920	4.4020	1.00
	2	5.60	0.3180	3.3008	2.00
	4	5.85	0.2540	5.3180	4.00
	6	5.80	0.2700	5.3180	3.66
	8	5.75	0.6480	5.8688	4.50
	10	5.85	1.1880	11.7376	5.50
	12	5.95	2.1840	20.7242	7.00
	14	5.85	2.6520	20.3574	7.00
	16	5.80	3.1680	32.2784	3.00
	18	6.05	3.9600	24.2088	5.00
2.	0	5.40	0.2560	10.2956	1.00
	2	5.80	0.4200	6.9863	1.50
	4	----[1]	0.3280	6.2509	6.33
	6	5.90	0.5280	7.3540	3.33
	8	5.90	0.9360	12.5018	4.00
	10	5.95	1.2960	11.3100	3.00
	12	5.90	1.9080	13.2372	3.00
	14	6.05	2.7280	26.1067	1.66
	16	6.10	3.4100	41.1824	1.50
	18	----	----	----	----
3.	0	5.45	0.2700	4.2528	1.00
	2	5.60	0.3020	3.3668	2.00
	4	5.75	0.3640	2.4808	2.00
	6	5.90	0.6240	3.3668	2.00
	8	5.70	1.2000	4.4300	4.00
	10	----	----	----	----
	12	5.90	2.2560	7.6196	3.50
	14	5.90	2.8160	12.4040	5.00
	16	6.10	3.2340	12.5812	4.00
	18	6.10	3.1460	11.8724	3.00
4.	0	5.40	0.2620	3.2538	1.00
	2	----	----	----	----
	4	5.60	0.3180	3.0624	1.50
	6	5.70	0.6720	4.7850	4.00
	8	5.70	1.4160	3.8280	3.33
	10	6.00	1.3200	4.7850	4.66
	12	5.80	1.9360	9.7614	3.50
	14	6.00	1.8920	12.2496	3.00
	16	5.80	2.5960	14.7378	4.00
	18	5.80	3.1460	24.1164	5.00

Table 22 (concl.)

Trial	Days of incubation	pH of milk culture	Tyrosine equivalent (mg./ml.)	Free fat acidity	Desirability of odor
5.	0	5.40	0.2620	3.2538	1.00
	2	----	----	----	----
	4	5.60	0.3100	2.8710	2.00
	6	5.70	0.7200	5.9334	3.00
	8	5.80	1.1040	4.4022	2.66
	10	5.80	1.2000	5.9334	3.66
	12	5.70	1.8260	9.3786	3.50
	14	5.85	1.5620	8.8044	3.66
	16	5.60	2.4200	11.2926	4.00
	18	5.60	2.2220	14.9292	5.00

[1] Data not available.

Table 23. Action of proteolytic culture No. III in whole milk.

Trial	Days of incubation	pH of milk culture	Tyrosine equivalent (mg./ml.)	Free fat acidity	Desirability of odor
1.	0	5.40	0.2920	4.4020	1.00
	2	5.60	0.2960	3.8510	2.00
	4	5.75	0.2660	7.7020	4.50
	6	5.90	0.4620	9.7191	7.00
	8	6.05	0.4320	15.4056	8.00
	10	6.05	2.1840	27.6934	6.50
	12	6.10	1.6200	29.3440	3.00
	14	6.15	2.6400	26.2262	5.00
	16	5.90	3.6800	36.6800	4.33
	18	6.00	3.9680	43.2824	4.50
2.	0	5.40	0.2560	10.2956	1.00
	2	5.90	0.4080	6.2509	1.50
	4	----[1]	0.3360	26.1067	6.33
	6	6.00	1.9080	80.8940	5.66
	8	5.70	2.0160	142.6676	6.00
	10	5.70	2.5960	193.0425	8.00
	12	5.50	2.6840	186.4239	5.50
	14	5.60	2.6400	233.4895	5.00
	16	5.60	2.5080	254.8161	5.00
	18	----	----	----	----
3.	0	5.45	0.2700	4.2528	1.00
	2	5.75	0.2980	3.3668	4.00
	4	5.85	0.3140	3.0124	3.50
	6	6.10	0.6480	3.7212	5.00
	8	5.85	1.3800	5.6704	6.00
	10	----	----	----	----
	12	6.00	2.9760	6.9180	6.50
	14	6.10	3.3220	32.0730	7.00
	16	6.10	3.4100	39.8700	5.66
	18	6.15	3.3220	26.9344	4.00
4.	0	5.40	0.2620	3.2538	1.00
	2	----	----	----	----
	4	5.90	0.4480	4.5936	3.50
	6	5.85	1.6800	9.7614	4.00
	8	5.70	2.2200	47.8500	4.00
	10	5.90	2.1340	55.6974	6.33
	12	5.60	1.9360	83.2590	5.50
	14	5.65	1.9140	85.9386	5.33
	16	5.40	2.8160	85.3644	6.00
	18	5.40	2.2880	91.4892	6.00

Table 23 (concl.)

Trial	Days of incubation	pH of milk culture	Tyrosine equivalent (mg./ml.)	Free fat acidity	Desirability of odor
5.	0	5.40	0.2560	10.2956	1.00
	2	5.85	0.4020	7.3540	1.50
	4		0.3020	9.5602	5.33
	6	5.90	1.7400	61.0382	5.66
	8	5.80	1.8600	136.7844	7.00
	10	5.60	2.3320	182.3792	9.00
	12	5.55	2.9700	190.4686	6.50
	14	5.65	2.4200	160.3172	5.00
	16	5.55	2.6840	242.6820	5.00
	18	----	----	----	----

[1] Data not available.

Table 24. Action of proteolytic culture No. IV in whole milk.

Trial	Days of incubation	pH of milk culture	Tyrosine equivalent (mg./ml.)	Free fat acidity	Desirability of odor
1.	0	5.40	0.2920	4.4020	1.00
	2	5.65	0.3320	3.1175	2.00
	4	5.80	0.2620	5.6848	3.50
	6	5.80	0.2260	6.2349	4.00
	8	5.80	0.4080	5.1352	6.50
	10	5.80	0.4320	9.9036	6.00
	12	6.00	0.5880	27.1432	6.00
	14	6.00	0.5160	8.6198	4.50
	16	5.80	0.7650	5.8688	3.33
	18	5.90	0.6160	6.9692	4.50
2.	0	5.40	0.2560	10.2956	1.00
	2	5.80	0.4240	6.9863	1.50
	4	----[1]	0.4320	10.2956	4.33
	6	5.80	1.6200	30.8868	5.00
	8	5.90	1.8840	98.5436	6.00
	10	5.70	2.1780	173.1867	8.00
	12	5.65	3.1020	173.1867	6.00
	14	5.60	2.2880	183.1146	4.33
	16	5.50	2.8164	232.3864	5.00
	18	----	----	----	----
3.	0	5.45	0.2700	4.2528	1.00
	2	5.60	0.2400	2.8352	2.00
	4	5.70	0.3360	3.1896	2.50
	6	5.85	0.4320	3.5440	2.66
	8	5.80	0.5040	4.0756	4.00
	10	----	----	----	----
	12	5.80	0.8640	6.0248	3.50
	14	6.00	1.6500	9.9232	4.00
	16	5.85	2.4200	31.1872	5.33
	18	5.75	2.2880	44.4772	5.00
4.	0	5.40	0.2620	3.2538	1.00
	2	----	----	----	----
	4	5.70	0.3100	3.8280	3.00
	6	5.60	0.5160	3.2538	3.00
	8	5.65	0.6360	3.4452	2.66
	10	5.65	0.6480	4.0194	3.33
	12	5.75	0.7040	7.2732	3.00
	14	5.90	0.7480	5.9334	2.66
	16	5.80	1.2980	7.4646	4.00
	18	5.95	1.6060	17.6088	5.00

Table 24 (concl.)

Trial	Days of incubation	pH of milk culture	Tyrosine equivalent (mg./ml.)	Free fat acidity	Desirability of odor
5.	0	5.40	0.2560	10.2956	1.00
	2	5.80	0.2700	6.9563	1.50
	4	----	0.2880	7.3540	3.33
	6	5.60	1.5960	16.1789	3.33
	8	5.85	0.6120	11.7664	4.00
	10	6.00	0.7920	25.0036	4.00
	12	5.90	1.8120	45.2271	4.00
	14	5.80	2.0680	108.4715	4.66
	16	5.60	2.5080	203.7058	5.00
	18	----	----	----	----

[1] Data not available.

EFFECTS OF SELECTED PROTEOLYTIC BACTERIA ON CHEDDAR CHEESE RIPENING

by

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The capital required for the process of ripening Cheddar cheese is a large portion of the total capital needed by the cheese manufacturer. Any method by which the length of the ripening period could be shortened, would produce an economic gain to the manufacturer and ultimately a savings to the consumer. Many investigations have been directed towards the improvement of the Cheddar cheese ripening process, but little significant success has been reported. The present study was the initial work of a project involving the use of added bacterial enzymes as a means of accelerating the ripening of Cheddar cheese.

One hundred and twenty eight raw milk samples were obtained and from this group, 30 proteolytic bacterial cultures were isolated on skim milk-agar plates. In order to have cultures which would be active at the pH and temperature of Cheddar cheese ripening, the isolations and subsequent screenings of the bacteria were carried out in media which had been initially adjusted to pH 5.4 and were incubated at 10°C. Selection of the cultures for use in the study was based on their production of a Cheddar cheese-like odor in milk. It was observed that the cheese-like odor was produced in whole milk and not in skim milk. This observation supports the conclusion, by other workers, that milk fat is necessary for the production of desirable flavor in Cheddar cheese. Four cultures were selected from the group of 30 bacterial cultures for subsequent use in

the study. These cultures were subsequently identified as being members of the genus Lactobacillus.

The actions of the selected cultures in whole milk, which was initially adjusted to pH 5.4 and then incubated at 10°C., were studied. It was found that 36 to 54 percent of the variation in desirability of the Cheddar cheese-like odor produced could be explained statistically by protein degradation (tyrosine equivalent) and fat hydrolysis (free fat acidity). The seemingly low correlation may be because free fat acidity was not a suitable measurement of fat hydrolysis. In addition, the odor producing compound(s) may have been the results of reaction(s) subsequent to protein and fat decomposition.

Of the four cultures employed in the manufacture of cheese, one appeared to be promising as far as the use of whole milk cultures was concerned. This culture yielded a flavor and a body and texture in cheese which scored higher than that of the control cheese after 30 and 60 days of ripening. Upon longer ripening, the cheese containing this culture became slightly bitter in flavor and weak in body. As a result, this cheese scored slightly lower than the control cheese after 120 days of ripening. Statistical analysis of the data indicated that the mean flavor score (for the 120 day period) of the cheese containing this culture was equal to the mean flavor score of the control cheese. The mean flavor scores of the cheese containing the other three cultures were significantly

less than that of the control cheese. The predominant off-flavors produced in cheese by all four cultures were bitter and fermented. The fact that three of the cultures did not produce desirable flavor when added to cheese in whole milk cultures does not eliminate the possibility that enzyme preparations from any of the four cultures may have a desirable effect on Cheddar cheese ripening under the proper conditions.

Preliminary work in preparing crude enzymes from whole milk bacterial cultures was carried out. It was found that the bacterial enzyme systems were separated from the milk culture with the precipitation of the casein at its isoelectric point.

It was recommended that separation of enzyme systems from the bacteria employed in this study and their subsequent use in cheese manufacture should be investigated. It was also recommended that lipolytic bacteria should be isolated from raw milk and then their effects on Cheddar cheese ripening subsequently studied.