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Proteolytic Enzymes of Lactic Streptococci and Their Use in Improving Body and Texture of Direct Acid Cottage Cheese

Gulamnabi Y. Vahora

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PROTEOLYTIC ENZYMES OF LACTIC STREPTOCOCCI AND
THEIR USE IN IMPROVING BODY AND TEXTURE
OF DIRECT ACID COTTAGE CHEESE

by

Gulamnabi Y. Vahora

A thesis submitted in partial fulfillment
of the requirements for the degree
of
DOCTOR OF PHILOSOPHY
in
Bacteriology and Public Health

Approved:

UTAH STATE UNIVERSITY
Logan, Utah

1973
ACKNOWLEDGMENTS

I owe much to Dr. C. A. Ernstrom, my Thesis Director, and F. J. Post, my major professor, and express my heartfelt deep sense of gratitude towards them for their keen interest and encouragement throughout the course of these investigations. Their valuable suggestions and constructive criticism during the preparation of this dissertation have been of immense value.

Sincere thanks are due to Dr. G. H. Richardson, Dr. V. T. Mendenhall and Prof. P. B. Larsen, for their constant encouragement and valuable suggestions during this work.

I would also like to thank Dr. L. W. Jones and Dr. C. A. Westby for their review of this manuscript and Mrs. Jean Post for her kind help in Statistical Analysis.

I shall be failing in my duty if I do not thank my friends, Mr. G. C. Chokshi, Dr. M. Habib, Mr. C. G. Kale, and Miss H. R. Patel and every individual in the department of Nutrition and Food Sciences, who cooperated with me in one way or another during the course of this work.

My deepest appreciation goes to my parents, brothers and sisters, whose incessant love has been a perpetual inspiration during my academic career.

Gulamnabi Y. Vahora
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ABSTRACT

Proteolytic Enzymes of Lactic Streptococci and Their Use in Improving Body and Texture of Direct Acid Cottage Cheese

by

Gulamnabi Y. Vahora, Doctor of Philosophy

Utah State University, 1973

Major Professor: Dr. F. J. Post
Department: Bacteriology and Public Health

Proteolytic activity of Streptococcus cremoris HP, P₂ and AM₂; Streptococcus lactis-C₂, ML₈ and a commercial mixed culture was determined in skimmilk and whey by measuring the increase in Absorbancy at 274.5 nm of a pH 4.6 soluble filtrate, following incubation in a 2.5% casein solution. Proteolytic activity, rate of acid production and growth rate decreased in order of C₂, HP, mixed culture, AM₂, ML₈ and P₂ at a regulated pH (6.1 - 6.2) and 32 C. Maximum proteolytic activity for all cultures was found during the stationary phase of growth. Extracellular protease activity was purified 1000 fold from S. lactis-C₂. Enzyme activity was stable at 80 C for 20 min and lost 41% of its activity after 80 min. A linear increase in activity was observed between 25 and 50 C after 5 hr incubation at pH 6.2.

Partial culturing of skimmilk, and incubation of a freeze-dried crude enzyme preparation in skimmilk prior to acidification was used for Cottage cheese
making. Partial culturing or incubation of freeze-dried crude enzyme preparation in skimmilk prior to manufacture of direct acid Cottage cheese, increased the moisture and firmness of the finished curd, and significantly improved the body and texture of the creamed product. Partial culturing of skimmilk prior to acidification was also successfully used for the continuous process where good Cottage cheese curd was also obtained.
INTRODUCTION

Cottage cheese is an easily digested high-quality protein food which is becoming increasingly popular. Home economists have devised hundreds of recipes using Cottage cheese, chiefly in salads, desserts, and to a lesser extent, in preparing cooked dishes. Low fat (2-4%) and relatively high protein (12-14%), calcium, and vitamins (vitamin A, riboflavin, niacin and thiamine) make Cottage cheese highly valuable from a nutritional point of view and these qualities are emphasized in promoting and advertising, especially to the diet-conscious consumer. Cottage cheese sales are directly related to the quality of the product. Total sales and consumption would increase if variations in quality were eliminated, thus providing a more uniform product throughout the industry (40, 109).

In recent years, Cottage cheese has moved from its position as a by-product, manufactured solely as a means of disposing of surplus milk, to the status of a major dairy product. Prior to commercial production of Cottage cheese, surplus skimmilk was used as an animal feed or was discarded as waste. Now more than 19% of the total skimmilk in the United States is used to manufacture Cottage cheese. The production and consumption of Cottage cheese in the United States has increased over the past several decades from 70 million pounds in 1920 to over 1 billion pounds in 1971 (110, 120). Per capita consumption has increased from 2.6 pounds in 1940 to 4.8 pounds in 1970 (108). As the population
and per capita consumption continue to increase, it becomes more and more imperative to improve and design new techniques and equipment to ensure efficient production of a uniform high quality product.

Cottage cheese is conventionally made from skim milk by addition of a starter culture with or without the addition of rennet. In conventional Cottage cheese making, the activity of a starter culture plays a very important role. But making cheese with starter is a slow process and there are very few opportunities for mechanization. With the conventional method, producers depend exclusively upon the activity of starter cultures, even with the trend toward labor-saving automation. The most serious problems in the manufacture of cheese by this process are related to abnormal or erratic behavior of the starter cultures. Slow acid production, agglutination of starter organisms, excessive gas production accompanied by floating curd, absence of flavor and aroma or abnormal flavor, and in some instances complete inhibition of acid development result in loss of the skim milk (40, 107, 109).

Several workers have tried to overcome these problems by making cheese curd by direct addition of acid. Patents by Ernstrom (41, 44) have provided a solution to the problems associated with conventional Cottage cheese making. However, the body and texture of curd from direct acid Cottage cheese were not quite as good as that manufactured by the conventional process. Direct acid cheese curd lacks the firm meaty texture of conventional curd and tends to be mealy and soft (62). Consumer acceptance of direct acid Cottage cheese could be increased if the body and texture were improved. Direct acid cheese making
could eliminate many problems associated with the conventional cheese making process, and provide a uniform product for the consumer.

This study was designed to investigate ways to improve the body and texture of direct acid Cottage cheese curd, and to improve the commercial potential of the continuous direct acid process. An attempt was made to determine the extent of growth of lactic streptococci in skim milk required to produce a good-bodied Cottage cheese curd. Other goals included the development of testing procedures for measuring the proteolytic activity of lactic streptococci growing in milk and whey, determine the optimum condition for production of proteases, and correlate bacterial protease concentration with improved body and texture of the cheese curd.
Manufacturing Procedures for Making Cottage Cheese

Cottage cheese manufacturing procedures have changed radically during the past thirty to thirty-five years. Small grain curd Cottage cheese with a sour flavor has disappeared from most metropolitan markets and a mild flavored cheese with a soft, meaty texture has appeared (40, 109, 120). This new product has become a food staple in this country with a current annual consumption of over one billion pounds. Manufacture of Cottage cheese involves many problems, and a product of uniform quality is not easy to obtain (4). The usual methods of manufacture require a long sixteen hour set or a short five hour set. A recently developed method of forming curd by direct acidification can save time by avoiding the setting period. Conditions and facilities in each plant vary with the method used. All manufacturing methods require that cheese makers be consistent in repeating their procedure (120).

Cottage cheese making with starter

Lactic acid produced by starter bacteria coagulates casein, and aroma compounds produced by associated organisms give Cottage cheese its pleasant flavor (4). Good Cottage cheese cannot be made by conventional methods without a good culture. Manufacturing methods vary chiefly in temperature of setting,
amount of starter, and amount of rennet used. Procedures differ also in the size of curd, creaming rates, type of cream used, degree of Cottage cheese flavor and added flavoring condiments (40, 120).

Conventional Cottage cheese should have good body, texture and flavor; attributes which are demanded by the consumer. Its manufacture requires care, labor, time and money. Making Cottage cheese with starter cultures is a slow process and there are rare possibilities for mechanization. Even with trends toward automation, the conventional method is still dependent upon the activity of the starter for acid formation (71, 107).

Sometimes for unknown reasons there is a starter failure, resulting in a slow or complete absence of acid production. Bacteriophage may inhibit culture bacteria, or agglutination by some strains of starter organisms in the cheese vat, may cause sludge formation at the bottom of the vat. Such problems have rendered conventional Cottage cheese making expensive and laborious. A lot of attention by highly technical personnel is necessary. There is no question, however, that this procedure results in a good product.

Cottage cheese making without starter

Increased per capita consumption of Cottage cheese in the United States will require a more practical and efficient method of production. Several systems have been patented with claims for continuous production of cheese curd. Harper and Seiberling (56) described a system to produce cheese curd on a continuous basis. No apparent use has been made of this system and a study of the
patent diagrams indicate the system would be extremely difficult to sanitize.

Another patent of Spiess and Hollis (102) describes a method and an apparatus for the continuous production of cheese curd which is of more sanitary design. These studies suggest that a continuous process will probably depend on a direct acidification technique.

In an effort to avoid the problems of non-uniform curd, bacteriophage, antibiotics in the milk, highly heterofermentative cultures and expenses involved in carrying starter, several attempts have been made to manufacture cheese by non-bacteriological methods (9, 13, 31, 42, 51, 55, 64, 70, 84, 97). There are two general approaches to non-bacteriological cheese making:

(1) The addition of compounds which undergo hydrolysis in aqueous solution to produced acid;

(2) The direct addition of acid to skimmilk.

Acidification by ester hydrolysis. Mabbitt et al. (78) embodied the two approaches by using lactic acid or hydrochloric acid (HCl) in combination with D-glucono-delta-lactone in Cheddar cheese making. The cheese thus produced had a crumbly body and lacked typical Cheddar cheese flavor. A good quality Cottage cheese of bland flavor was made by Dean and Hammond (28) using D-glucono-delta-lactone and meso-lactide as acidifying agents. Cheddar cheese and sour cream-like products are also manufactured by using D-glucono-delta-lactone (29, 30).

The major disadvantages of this process for commercial Cottage cheese making were in the high cost of the acidulants and the long coagulation time which has limited its use in a truly continuous operation (48).
Acidification by addition of organic or inorganic acids. Early efforts to add acid to skimmilk in an attempt to duplicate the pH achieved by bacterial fermentation has proved unsuccessful as a solution to overcoming the problems associated with conventional Cottage cheese making (94, 105, 118). The reason was: Unlike the conventional process, where the acid is formed in situ by bacteria, agitation was necessary to get the added acid intimately mixed with the milk, and agitation at the time of coagulation prevented the formation of a curd (13, 83, 102, 106).

The discovery that milk at refrigeration temperature could be acidified to the isoelectric point of casein without causing precipitation led to successful direct acidification (42, 70). Spiess and Hollis (12) suggested that skimmilk at 0-4.4 C could be acidified to pH 4.4 to 4.6 by addition of acid to a stream of milk. They proposed that acidified milk be warmed to 32 C until coagulation was completed. The degree of success or failure with this automated procedure was not indicated.

Ernstrom (42) as early as 1954 noted that, during the low-temperature precipitation of casein from skimmilk, concentrated lactic or hydrochloric acid could be added at or below 4.4 C without causing localized precipitation or spot coagulation. He succeeded in producing a smooth, firm curd by warming the acidified skimmilk by electrical resistance heating while maintaining it in a quiescent condition (42). Ernstrom later stated (44) that satisfactory direct acid curd could be formed with lactic acid, phosphoric acid, acetic acid, hydrochloric acid and sulfuric acid. Sulfuric acid, however, was the least useful because it had to be
added in a very dilute form to prevent the generation of heat. For economic reasons he suggested the use of hydrochloric acid. Addition of acid to cold milk offers no particular problem when the milk solids content is between 8 and 16%. McNurlin (83) and Ernstrom (41) observed that slight agitation of preacidified skimmilk during pumping or during the initial stages of heating would be harmless if milk could be fortified up to 16% solids.

An automatic direct acid curd former was developed by the C. P. Division, St. Regis Company, Chicago, Illinois. When a small continuous stream of preacidified skimmilk (pH 4.6) was warmed in a tube to a regular coagulating temperature (32 C), curd was formed in the tube and cut into curd particles as it emerged from the tube, Figure 1. Cooking, whey-drainage and washing were done continuously and automatically (42, 48). Here, electrical resistance for curd formation was ruled out, because of its unsuitability for the commercial application (41, 44, 83). This machine was capable of manufacturing 2,000 lbs of curd per hour. If concentrated skimmilk was used, production could be increased. In comparison, it would require a plant with six 20,000 lbs vats to make the same amount of curd in one eight-hour day by conventional methods. This method of processing eliminates the use of cheese vats and has the advantage of a continuous, completely enclosed system for forming, cooking and washing the curd. Recently, standards for Cottage cheese were modified to permit the use of direct acidification procedures (19). Little and Coeur (70) claimed that acidified milk (pH 4.00 - 4.95) can be coagulated under cold conditions (4.4 - 15.5 C) by addition of excessive amounts of rennet and good
Figure 1. Operating principle of the continuous curd former (42).
quality Cottage cheese can be made from this milk.

The characteristic stretchiness and elasticity of rennet curd made by direct acidification of milk led Breene (13) and Breene et al. (14) to investigate the possibility of making Pizza or Mozzarella cheese in this manner. Their work showed that a quality Pizza cheese could be made by direct acidification (15). Ernstrom later modified the procedure for making Pizza cheese which is more adaptable to a continuous cheese making process (43).

Shehata and Olson (97, 98) developed a direct acid method for making Blue cheese, which represented a 50% saving in time over conventional processes. Their product was similar in body and texture to normal Blue cheese. It should be noted the Blue cheese is the only variety requiring ripening that has been successfully made by direct acid methods. This is probably due to the fact that this cheese depends upon mold growth, rather than upon the lactic starter, for flavor development.

**Firmness of Cottage Cheese Curd**

Using normal skimmilk inoculated with 5% lactic culture, without rennet and set as 32 C, Emmons et al. (37) found that curd firmness at the A-C (Acid Coagulation) end point (at pH 4.8) was approximately 2 to 4 grams. At that point the curd was quite fragile but still firm enough to cut. They also reconstituted non-fat dry milk (NDM) to about 17% solids. After addition of 5% culture, they measured curd firmness at the A-C end point after incubation at 32 C with
and without added rennet. On ml of rennet per 1000 lb of skimmilk resulted in a seven fold increase in the curd firmness over the unrenneted sample (38).

An increase in curd firmness with increasing acidity was noted by Heinemann (58) and later confirmed by Emmons et al. (39), who commented that acidity was probably more important in the development of curd firmness than time. The effect of starters on curd firmness is not well established. Even though firmness increases with increasing acidity, several workers (39, 57, 122) noted poor agreement between titratable acidity and curd firmness of coagulum formed by a number of starter cultures. Heinemann (58) attributed the lack of agreement to proteolytic activity of some of the cultures used in his study. Emmons et al. (39) discounted the importance of proteolysis when they found close correlation between pH and curd strength of the curd produced by 17 lactic cultures.

Unlike previous workers, Williamson and Speck (122) used unrenneted curd in their studies of several lactic cultures. They found no important relationship between proteolytic activity and curd firmness; a very close correlation between titratable acidity and pH, and poor agreement between titratable acidity values and curd firmness. The wide variation in curd firmness had many causes (37, 38, 58, 122). There seems to be general agreement that pH at cutting is more important than curd firmness in determining the characteristics of the final Cottage cheese curd, and that the pH at cutting should be about 4.6 to 4.7 with normal skimmilk (40, 83, 120). In contrast to this it was suggested by Kale (62)
that during setting of Cottage cheese milk, measurable proteolysis could contribute to the body and texture of Cottage cheese.

McNurlin (83) showed that firmness increased at all pH levels (pH 4.4, 4.6, 4.7, 4.8 and 5.0) with increasing setting temperature and with the length of time at which the curd remained at the setting temperature. At 32°C the curd appeared to be firm enough to cut at all pH values except at pH 5.0. The curd at 4.6 and 4.7 was generally firmer than that at pH 4.4, 4.8, or 5.0. However, none of the curd formed below 27°C was firm enough to cut.

Cottage cheese is a non-ripened cheese; therefore, in the direct acidification procedure it was assumed that the main function of starter was to produce enough acid from the fermentation of lactose to cause coagulation of casein (42). However, several workers have shown that cheese curd prepared by direct acidification was softer and often more mealy than the curd made with the starters (9, 62). These observations suggest that besides production of acid and flavoring compounds, starter organisms contribute to the body and texture of Cottage cheese curd.

**Cottage Cheese Starter**

Cottage cheese manufacturers recognize that a good lactic culture is the most important single contribution to successful cheese making. The addition to pasteurized skimmilk of a bacterial culture capable of producing lactic acid from lactose is the first step in the process of Cottage cheese making. Although
various coagulators containing the enzyme rennin frequently are used in the manufacture of Cottage cheese, skimmilk undergoes acid coagulation rather than rennin coagulation. For the successful manufacture of Cottage cheese, it is also important that skimmilk undergo acid coagulation in a quiescent state; therefore, the addition of lactic acid to the milk is not a satisfactory substitute for acid production by bacterial culture (40, 44).

The most common culture employed in Cottage cheese making consists of a mixture of lactic organisms capable of lactic acid production and citric acid fermentation. The acid producing organisms are either *Streptococcus lactis* or *Streptococcus cremoris* and the citric-acid fermenting species are either *Leuconostoc citrovorum* or *Leuconostoc dextranicum* (4).

Cottage cheese manufactured with both lactic acid-producers and citric acid-fermenters generally has more flavor, due to diacetyl and volatile acids, than Cottage cheese manufactured by lactic acid-producing organisms alone. However, Cottage cheese has been made successfully with a pure culture of either *S. lactis* or *S. cremoris*. Such cultures are more uniform in acid production from day to day, producing very little CO₂, and testing procedures for bacteriophage are simplified (20). However, Emmons et al. (39) reported variability among starter cultures in the relationship of titratable acidity of whey and pH of curd.

**Flavor**

In conventional Cottage cheese making, careful control is required to maintain uniformity of flavor. However, many of the desirable
flavor and aroma compounds are water soluble and are lost in the whey and wash water, regardless of care taken in the manufacture. Diacetyl, a major flavor component in Cottage cheese, varied from 0 to 3 ppm in a number of commercial Cottage cheeses, tested by Mather and Babel (81, 82). The use of heterofermentative mixed cultures that produced enough diacetly and other flavor components often resulted in excess CO₂ production and floating curd when organisms such as *Streptococcus diacetilactis* were present (104). Later attempts by Babel (4) and Collins (20) to obtain overall uniformity by homofermentative lactic cultures resulted in bland cheese.

The fact that the Cottage cheese need not be ripened makes this variety ideal to manufacture by direct acidification. The flavor components in conventional Cottage cheese differ widely from sample to sample (81, 107, 119). It was found that sufficient flavor could be imparted to a bland curd in several ways. Hales (53) proposed that starter distillate be added to intensify the flavor. This practice increased the flavor level to a point that is sometimes criticized for being unnatural. Another approach was the development of highly flavored cream dressings. One such product was made by Mather and Babel (82).

Skimmilk was heated to 87.5°C for 1 hr, cooled to 21°C and inoculated with 2 to 4% *S. citrovorus* culture. This milk was incubated for 24 hr at 21°C, acidified to pH 4.3 with a 15% sterile citric acid solution, then incubated at 21°C for an additional 24 hr. This mixture was used to standardize cream for Cottage cheese dressing to about 12% fat. Standardization of diacetyl to 1 to 2 ppm was
accomplished by varying the original fat content of the cream, thus varying the amount of skimmilk culture necessary to standardize the fat content of the creaming mixture. The flavor level in the creamed cheese remained constant for 8 to 10 days before it started to decrease. Lundstedt and Fogg (77) also suggested that medium to high aroma may be produced at temperatures between 0 to 7.4°C in creamed Cottage cheese by adding 0.25 - 1.0% of an 18 hr old citrated Cottage cheese whey culture of *S. diacetylactis* to the finished cheese without changing the pH of the cheese (76).

**Proteolytic activity**

Lactic cultures have been recognized primarily as saccharolytic organisms. However, the results of various investigations have shown that many strains are capable of causing protein degradation in milk (111, 113, 114, 116, 124). Among the proteolytic strains, there are wide variations in activity (49, 52, 54, 61, 62). Early studies showed definite proteolytic properties by *S. lactis* and *S. cremoris* while some apparently do not. Kelly (63) compared proteolytic action of *S. lactis* and *S. cremoris* in milk with and without added calcium carbonate. The carbonate increased hydrolysis of both protein and sugar. Hammar and Babel (54) reported that in milk with added calcium carbonate, *S. lactis* from various sources decomposed more protein at 14°C to 20°C than at 36°C during 2 to 4 months. This proteolytic activity of lactic cultures was due to the endocellular and extracellular proteolytic enzymes. These enzymes play a very important role in curing various types of cheese, e.g., Cheddar and Roquefort (47, 60, 90, 91).
Intracellular, extracellular and particle bound activities have often been described and there are possibilities for several different enzymes as well as one or a few of low specificity. It has been observed that nearly all proteolytic enzymes clot milk under proper conditions (45). The so-called "Sweet curdling" of milk is attributed to milk-clotting enzymes secreted by bacteria (45).

A proteinase secreted by a group A Streptococcus has been investigated by Liu and Elliott (73, 74). Under ordinary culture conditions, it has been found that activation of the zymogen is dependent on both proteolytic activity and reduction by the bacterial cell envelopes. This proteinase, like several of bacterial origin, exhibits a broad side chain specificity (33, 49, 75, 115).

Intracellular proteinases from several bacteria were measured by Vedehra and Boyd (111). There was a common proteolysis optimum between pH 6 and 7, but S. lactis also showed a second peak at pH 5.5. Lactobacillus casei and Lactobacillus bulgaricus were found to produce endocellular proteolytic activity, whereas S. lactis secreted an extracellular enzyme at pH 7 (6). The presence of extracellular proteolytic activity from S. lactis was also observed by Vander Zant and Nelson (113, 114). Williamson, et al. (123) reported 120-fold purification of a protease enzyme from the cell free extract of S. lactis. Optimum pH was 8.5 and enzyme was stable to heat treatment. It was inhibited by p-chloro-mercuric benzoate, but not by di-isopropyl fluorophosphate, indicating a requirement of free sulfhydryl groups for activity.
Sato and Ohmiya (95) sonicated cells of L. bulgaricus, L. casei, S. lactis and S. cremoris and measured their pH and temperature optima. According to their interpretation, there might have been two enzymes in the extracts. Bradsaeter and Nelson (11) sonicated the cells of L. casei grown in a protein medium. The supernatant showed highest proteinase activity against casein at 50°C, but at the same time the pH-optimum was displaced from 6.5 - 7.0 at 30°C to pH 5.5 - 6.0 at 40 to 50°C. They also observed some characteristics of peptidase activities from the same organism (12).

Cowman and coworkers (21, 22) published a series of papers on low temperature storage of lactic starter cultures and more particularly on proteinase from S. lactis. Storage of S. lactis at 3°C resulted in slightly reduced viability after 10 days. The residual proteolytic activity in milk decreased markedly when cultured in milk at 22°C. Acid production was also slow (21). Addition of pancreatic extract to the stored cells completely restored acid production at 22°C. Comparable experiments were made by storing cells at -20°C and -196°C for sixty days (22). At -20°C viability decreased gradually almost to zero and acid production was reduced to 10 to 20%. Residual proteinase activity underwent a sudden drop within the first three days, whereas the rest was rather stable to storage until the sixteenth day. At -196°C rather small effects were observed on all three characters measured.

The properties of a membrane bound and an intracellular proteinase from S. lactis were also reported (23, 24, 25, 26). The intracellular enzyme was inhibited by p-hydroxy mercuric benzoate (PHMB). But there was no effect of
reducing agents, ferrous ions or magnesium ions; and it was stable to storage at 3 C. On the other hand, membrane-bound proteinase was not affected by PHMB and was labile to storage at 3 C. It was also activated by ferrous and magnesium ions and by cysteine. Both proteinases were purified by column chromatography on Sephadex G-50, DEAE-Sephadex and Sephadex G-100. They were subjected to a reversible temperature-dependent association-dissociation equilibrium. Associations were favored at 3 C giving first a dimer and gradually trimers and higher polymers on storage. Also, variations of pH, ionic strength and concentration altered the equilibrium. Crystallization of the intracellular protease was mentioned without detail.

Smittle and Koburger (99) stored *S. lactis* in phosphate buffer, 0.05 M of pH 4.5 and 8.5 at 4 C for ten days. At pH 4.5 acid producing ability, viability and residual proteinase activity decreased gradually whereas ability to synthesize extracellular proteinase on recultivation first increased through 4-5 days but fell quickly thereafter. Acid production per cell fell, but was more than restored within ten days. Storage at the higher pH resulted in a small decrease of viability and acid production, the same drop in residual proteinase activity as when stored at pH 4.5. Gradual decrease of proteinase synthesizing ability and some changes in acid production per cell were noticed. They concluded that high, extracellular proteinase activity was not necessary for acid production and that the changes in proteinase synthesis at pH 8.5 were caused by endogenous activity allowing a more pronounced metabolism, in contrast to cells at pH 4.5 which cannot repair aging damage.
Soluble nitrogen values of protein degradation determined by the Kjeldahl method after tichloroacetic-acid precipitation of protein and, by measuring tyrosine-tryptophane using Folin-Ciocalteu method, indicated good parallelism of protein degradation when used on milk cultures of S. lactis (46, 114). It was also shown that considerably more soluble nitrogen and tyrosine-tryptophane were produced in cultures when the pH was controlled within the range pH 6.0 to 7.5 than when pH was not controlled (6). Supernatant from sonicated cells were studied for proteolysis, and different culture media were tried. Extracts from cells grown in protein media produced the highest tyrosine values. The heat-lability of the proteinase and adaptive function of the enzyme system was also mentioned (113). Incubation of S. lactis in milk revealed increasing numbers of peptides: 3 at zero hour, 5 at 24 hr and 6 at 96 hr. S. lactis showed some peptidase activity which was optimum between pH 7.0 and 8.5 and had rather high heat stability (115, 116).

Several studies have attempted to determine the breakdown fractions from protein in milk and cheese caused by lactic acid bacteria. Not all of this work will be considered in detail as they primarily aid in understanding the flavor problems in dairy products, stimulation affects of culture media, as well as the evaluation of the products in nutrition. Their interpretation in bacterial enzymology is not simple, bearing in mind the complex systems used. Some recent publications may be useful for references (10, 17, 32, 52, 60, 68, 92, 93).

Elucidation of protein degradation processes during cheese ripening has been tried by following the electrophoretic patterns of casein obtained by the
moving boundary and polyacrylamide gel technique (68, 69, 71). By using these methods it was possible to determine which of the casein fractions were attacked by proteinase from *S. faecalis* variety *liquifaciens* and in what order. Both α and β-caseins were attacked but β-fractions disappeared first (68).

In a study by Annibaldi (2) electrophoretic patterns of casein were examined to compare the attacks by several bacteria. *L. helveticus* degraded the αs and β-casein at nearly the same rate as *S. faecalis* started degrading the β-fraction. *Alcaligenes* and *Bacillus subtilis* attack first β-casein and then the α-fraction. A few strains out of 200 thermophilic lactobacilli were highly proteolytic (10). There was no relationship between acid production and protein degradation, but casein breakdown occurred under an acid condition.

Japanese workers have tried to shed light on protein degradation by lactics through the study of the changes in casein fractions by gel electrophoresis, ion exchange chromatography and light scattering (86, 87, 88). *S. cremoris* was shown to hydrolyze α-casein more than β-fraction, whereas *L. bulgaricus*, *L. helveticus* and *S. lactis* attacked α-casein but not β-casein. The action of proteases on casein from cultures of these organisms revealed varying degrees of hydrolysis indicating the presence of different bacterial proteases.

Garvie and Mabbitt (50) raised the acid production of a slow *S. cremoris* to that of a fast strain by addition of peptone but they did not observe any difference in the growth rate. They concluded that the change from a fast to a slow strain on continued transfer is due to the loss of ability to utilize the nitrogen compounds in milk. The possible importance of peptides for the growth of *L.*
casei as well as the mechanisms for the uptake of amino acids compared to peptides were studied by Leach and Senll (66, 67). They reported at least two different mechanisms.

During the ripening of Cheddar cheese, a great share of enzymes for protein degradation was of bacterial origin (90, 91). Characteristics of extracts from one year old Cheddar cheese and of S. lactis, L. casei and Micrococcus freundichii were analyzed by Baribo and Foster (5). Protease in extracts from Cheddar cheese and from S. lactis both showed two pH optimum: one at about 5.5 and another at about pH 7.5, when assayed at 30 C. Milk was incubated separately with different strains of S. lactis as well as one strain of Escherichia coli and one of Aerobacter aerogenes at 30 C. Soluble nitrogen in the supernatants was analyzed by the Kjeldahl method. Lactic culture activities all resulted in a rapid increase in soluble nitrogen which then continued more slowly. The other organisms showed only a little activity for the first period but produced a sudden increase between 7 and 15 days.

S. faecalis has been isolated from Cheddar cheese and the ripening effects considered (47). Bleiweis and Zimmerman (7) suggested that a proteinase from this bacterium was a zinc-enzyme. Somkuti (100) isolated a strain of S. faecalis var. liquefaciens from Cheddar cheese which secreted a proteinase maximally in the logarithmic phase when grown in a semi-synthetic medium. It was purified 193 fold. Seidel and Zimmerman (96) reported a cytoplasmic peptidase of this coccus which appeared to be of sulfhydryl type. It hydrolyzed
lysine-p-nitro anilide, one of several differences from the extracellular proteinase.

Bitter flavor may develop in Cheddar cheese if the pH is above 5.2 in the product and also if the salt concentration is low. High rennet activity resulting in the accumulation of polypeptides and a comparatively slower breakdown of casein by bacterial enzymes was suggested as an explanation by Czulak and others (27, 34, 35, 65). They concluded that unhydrolyzed peptides were causative, and that the existence of these substances in unexpected amounts could be traced to deficiencies in the enzyme system of the strain of the starter culture. In addition to bacterial enzymes, rennet is also active in cheese ripening and it has its optimum activity against para-casein at pH 5.2 in the presence of sodium chloride (104). Stadhouders (103), by using the larger quantities of rennet in cheese production, also obtained a higher frequency of bitter flavor. He concluded that rennet and milk enzymes were of limited importance in the breakdown of proteins in Dutch cheese and that, although rennet stimulated the production of amino acids, presumably by bacterial enzyme activity, it did not liberate these acids.

Partial Culturing and Use of Bacterial Enzymes

for Direct Acid Cottage Cheese

In the process of continuous Cottage cheese making without using bacterial starter, we sacrifice the typical cultured product flavor and the loss of body and texture of Cottage cheese curd desired by most consumers (40, 59, 62). On the
other hand, many problems in conventional Cottage cheese preparation are related to the use of bacterial culture as mentioned earlier (40).

Problems related to the direct acid and the starter method could be solved if some means could be found to use principles involved in both the procedures. The utilization of the direct acidification along with exponential-phase noncoagulated starter culture might serve the purpose. The direct acidification method will save labor and time which is an important factor from an economical point of view; whereas, exponential-phase culture will provide essential enzymes for fermentation of lactose, production of pleasant flavor and a firmer body and texture of Cottage cheese.

The combination of a starter and chemical acidulant was suggested by Boddicker et al. (8). Two normal HCl was added to the reconstituted skimmilk of 10%, total solids at 5 C. After addition of 5% lactic starter, the milk was warmed to 31 C and held until coagulated (pH 4.7). A 25% reduction in the time needed to reach pH 4.7 was observed by acidification of skimmilk to pH 6.0 and 5.75. Acidification to pH 5.0 resulted in a 50% reduction in time. But shattering of the curd was reported upon cutting. Curd tension formed by partial culturing was less than the controls when skimmilk was acidified to pH 5.5 and 5.6.

Bristol and Martin (16) studied the partial culturing of acidified skim-milk for Cottage cheese making by exponential phase cultures. A noncoagulated exponential phase culture was prepared by inoculating skimmilk with 0.5% or 1.0% of a commercial starter and incubating at 21 C for 15 hr or at 32 C for 5 hr. Skimmilk was pre-acidified by addition of citric acid, phosphoric acid
or a commercial mixture of the two acids. As compared to the conventional procedure, partial culturing showed a 35 to 52% reduction in setting time. A comparison of the physical appearance and aroma of Cottage cheese curd produced by partial culturing indicated similarity to curd produced by the conventional procedure. But it was less firm and did not break away clearly from the sides of the cheese vat (16).
MATERIALS AND METHODS

Chemicals and Media

Hydrochloric acid, ammonium sulfate and ammonium hydroxide were reagent grade. Lactivate powder (phosphate yeast stimulant blend) was obtained from Dr. G. H. Richardson (Department of Nutrition and Food Science, USU). Dry whey powder (food grade) was obtained from Cache Valley Dairy Association, Smithfield, Utah, and non-fat dry milk (NDM) (low heat, grade A) was obtained from Hiland Dairy Association, Murray, Utah. DEAE-Sephadex-A-50-120 (G-50) was purchased from Sigma Chemical Company and casein was prepared from skim milk using the method of Van Slyke and Baker (117). Raw skim milk for the production of Cottage cheese was obtained from the Utah State University Dairy Products Laboratory and was pasteurized at 63°C for 30 min.

Growth of Bacteria

Strains of lactic streptococci employed in the studies were S. lactis, strains C_2 and ML_8; S. cremoris, strains HP, AM_2, and P_2, and a commercial mixed culture. Strains C_2 and HP were obtained from Dr. D. B. Emmons (Canada Department of Agriculture, Ottawa, Canada); P_2, AM_2 and ML_8 strains were obtained from the New Zealand Dairy Research Institute. Cultures were maintained in 10% sterile reconstituted NDM. Transfers were made following 16 hr at 21°C and stored at 4°C. A non-sludging high protease producing strain
of *S. lactis* (strain C₂) was the main organism used for the enzyme preparation and in cheese making. The enzyme was prepared by growing *S. lactis*-C₂ in 10% reconstituted sterile NDM and also in a sterile whey medium using 5% inoculum. The whey medium was composed of 70 g powder whey, 3.219 g Lacto- tivate powder (as a stimulant), and 1000 ml of distilled water, sterilized at 121 C for 15 min.

Organisms were allowed to grow in a 4 liter Virtis magnetic-drive fermenter regulated at a pH of 6.1 to 6.2, and at a constant temperature of 32 C, Figure 2. Control of pH was accomplished by a slightly modified Heath Recorder and Autotitrating Syringe using 20% (V/V) NH₄OH solution. Regulation of pH was also adjusted manually when necessary. At intervals of one hour, samples were aseptically drawn from the fermenter to determine bacterial counts and enzyme activity. Bacterial counts were carried out by the pour plate method using sterile nutrient agar (Difco) with 0.5% NDM powder. Plates were incubated at 30 C for 24 to 48 hr. Samples for enzyme assays were stored at -20 C until they were used.

After twenty-three hours the inoculated culture media showing the greatest enzyme activity was freeze dried on a Virtis model lyophilizer. The resulting powder was transferred to a glass-stoppered bottle and stored at -20 C.

**Purification of Proteolytic Enzymes**

A crude preparation of enzyme from *S. lactis* C₂, grown for 23 hr in a whey medium, was used in the purification scheme. Initial purification
Figure 2. Virtis magnetic-drive fermenter, Heath pH recorder and Sage Instruments infusion pump.
techniques, with some modifications, followed the procedures employed by Cowman et al. (26), in the purification of protease enzymes from lactic streptococci. All steps were carried out at 4 C in buffers containing 2.0 mM, 2-mercaptoethanol unless otherwise stated.

Cell free preparation

Two-hundred-seventy-two milliliters of crude cultured whey media prepared as described above were centrifuged at 27000 G for 30 minutes at 3 C. After centrifugation, 250 ml of supernatant was obtained. The supernatant was made 2.0 mM with respect to 2-mercaptoethanol (Fraction I).

Ammonium sulfate fractionation

Two-hundred-fifty-milliliters of supernatant obtained as described above were fractionated with 60.75 g (NH$_4$)$_2$SO$_4$ to produce 40% saturation. The 0-40% fraction lacked proteolytic activity, however, substantial activity was present in the fractions of 50%, 60%, 70% and 75% (NH$_4$)$_2$SO$_4$ saturation. These fractions were dialyzed for 10 hrs against 4.5 liters of 0.005 M sodium phosphate buffer, pH 6.2, to remove the (NH$_4$)$_2$SO$_4$ (Fraction II).

DEAE sephadex chromatography

A 25 ml portion of the 40-75% dialyzed (NH$_4$)$_2$SO$_4$ fraction was placed on a DEAE Sephadex (A-50-120) column (2.5 x 25 cm) previously equilibrated
with 0.005 M sodium phosphate buffer, pH 7.0 at 4°C. The column was washed with 20 ml of the same buffer followed by a 600 ml stepwise gradient of decreasing pH and increasing molarity. The gradient was generated with a gradient mixing device, having two side-by-side chambers of equal diameter. The mixing chamber initially contained 300 ml of 0.005 M sodium phosphate buffer pH 7.0 and the reservoir chamber, 300 ml of 0.05 M sodium phosphate buffer pH 6.0. Ten ml fractions were collected from the column after 45 ml of liquid, lacking in enzyme activity had been eluted. When 100 ml more had eluted, the reservoir chamber was made 1 M with respect to NaCl (13.61 g). Following this, eluates were collected as before. From each fraction 0.2 ml and 0.8 ml samples were removed for protein estimation and enzyme activity. The fractions demonstrating the highest activity (tube no. 15-20, first peak; and tube no. 40-43, second peak fraction) were pooled, Fraction III.

**Enzyme Assay**

Proteolytic activity of the enzyme was assayed by a modification of Valkaleris and Price (112). Casein at 2.5% solubilized in 0.1 N NaOH and adjusted to pH 6.2 was used as a substrate. It was stable on cold storage (4°C) for at least one week. This solution was tempered at 32°C for 15 min or more before use. A typical incubation mixture contained 10 ml of 2.5% casein (pH 6.2) solution and 0.5 ml of the enzyme preparation (10 μg protein) in a final volume of 12 ml. To test and control tubes in the thermostatically controlled
waterbath, enzyme preparation was added at 30 sec intervals. Under standard condition 10 ml of the casein solution was pipetted into all assay tubes. After 5 hrs incubation at 32 C, 1.41 N chilled HCl solution was added for deproteinization until the pH of the assay mixture reached 4.6 to 4.7. A final volume of 12 ml was achieved by addition of distilled water. At the end of 5 hrs incubation, 10 ml of the same 2.5% casein solution, which was kept at 32 C, was added in the blank tubes, and these tubes were also deproteinized in the same manner as the assay tubes. All tubes were centrifuged at room temperature for 10 min at 7710 G. Supernatants were measured for optical density against the corresponding blanks on a DB-G-Spectrophotometer (Beckmann Instruments Inc.) by scanning through the 200-300 nm wave length region.

Cottage Cheese Making

For comparative study Cottage cheese curd was manufactured by the conventional procedure and by direct acidification following partial culturing or addition of bacterial enzymes to the milk. In all these procedures curd was cut with 6.35 mm knives and cooked to 62 C in 120 min for one set of experiments and 59 C in 120 min for another set of experiments. At these temperatures, curd was held for 15 min and then washed and drained. The curd was washed twice with water at 20 C and 8 C respectively and kept in contact with each wash for 10 min in one set of experiments and 15 min in another set of experiments. The last wash-water was acidified to pH 5.0 with HCl (13.67 N).
Conventional procedure

The short-set method for Cottage cheese making as described by Emmons and Tuckey (40) was modified to include heating the curd and whey in a plastic box by electrical resistance (Figure 3), as described by Kale (62). The skim-milk at 32 C was set with 5% starter and 1 ml rennet per 1000 lb of skim milk. The curd was cut at the A-C end point (40), and was cooked and washed described above.

Direct acid method

The method described by Ernstrom (44) was used for cheese making. The curd was formed by acidifying the pasteurized cooled skim milk to pH 4.6 with concentrated (13.67 N) chilled HCl, and warming this acidified milk by electrical resistance to 32 C under quiescent state. After coagulation, the curd was cut, cooked and washed as described earlier.

Partial culturing and direct acidification

Five percent starter was added to pasteurized skim milk and the organisms were allowed to grow at 32 C until the pH of the milk reached 6.0 or 5.5. This milk was then immediately cooled to 4 C by circulating salt-brine (-1 C) through cooling coils immersed in the milk, Figure 4. The cold cultured milk was then
Figure 3. Apparatus for warming acidified skim milk and Cottage cheese curd by electrical resistance without agitation.
Figure 4. Apparatus for cooling partially cultured skimmilk by circulating salt brine through cooling coils immersed in the milk.
acidiﬁed to pH 4.6 with concentrated HCl, and Cottage cheese curd was made as described earlier.

**Use of bacterial enzymes and direct acidification**

Crude enzyme powder as described previously (p. 27) was used at the rate of 5%, 2.5%, 1% and 0.5%, which corresponds to 30 g, 15 g, 6 g, and 3 g for NDM milk medium and 21 g, 10.5 g, 4.2 g, and 2.1 g for whey medium, when added to 6 liters of skimmilk. This powder was dissolved in skimmilk at 32 C and held for 3 hr, 2 hr, and 1 hr at that temperature. At the end of the incubation time, milk was cooled to 4.4 C and manufactured into direct acid cheese as described.

Two sets of experiments were carried out at different times. In the first set of experiments, Cottage cheese curd was made by 4 different methods: Direct acidification, partial culturing to pH 6.0, partial culturing to pH 5.5 and by the conventional procedure. Curd was cooked by raising to 62 C in 120 min and was kept in contact with wash water for 10 min. In a second set of experiments, cheese curd was also made by 4 different methods: Direct acidification, conventional procedure and using 1% and 0.5% enzyme preparation. But in contrast to the first set, cheese curd was cooked to 59 C in 120 min and was kept in contact with wash water for 15 min rather than 10 min.
Continuous Cottage cheese manufacture

Milk was incubated for partial fermentation to pH 5.5. The cold cultured milk (pH 5.5) was then acidified to pH 4.6, 4.67 and 4.7 with concentrated HCl. In some cases various amounts of rennet were also added (at the rate of 0.5 - 2.0 ml per 1000 lbs of skim milk). Cottage cheese curd was made by the method of Ernstrom using the curd former (42). Curd was cooked to 65.6°C in 50 min and was in contact with each wash water for 15 min.

Moisture analysis

Moisture in Cottage cheese was determined by the A.O.A.C. method (3) for determining the moisture content of soft cheese.

Cream dressing

Cream dressing was prepared according to Manus (80).

Curd firmness

Curd firmness was measured according to the method of Emmons and Price (36). Curd was packed into a 5 cm stainless steel slotted cylinder closed at the bottom, Figure 5. The slots were covered with two side covers while a 5 lb weight pressed the curd for approximately 10 sec in order to pack it firmly. Free whey, but not curd, could escape via the covered slots. The cylinder was
Figure 5. Cottage cheese curd tension meter showing stainless steel cylinder and wire cutter.
placed on a scale, and a wire cutter was driven through the curd at a rate of 5 cm per 15 secs so it did not touch the container. Curd firmness was measured in grams.

Organoleptic evaluation of cheese

After the curd was creamed and allowed to stand for 24 hr, its body and texture was evaluated by a panel of judges on a 5 point scale: 1. superior, 2. excellent, 3. satisfactory, 4. objectionable, and 5. unsaleable. Average scores of the judges were reported for each treatment. Analysis of variance of the results of the scores was carried out according to the complete randomized design as described by Ostle (89).
RESULTS

Growth and Enzyme Production

Five single strains and one mixture of strains of lactic cultures were examined for growth rate, rate of acid production and production of proteolytic enzymes in reconstituted skimmilk and in fortified whey medium over a 24 hr period. Rate of acid production was measured by decrease in pH. It was noted that strains C₂, HP and the mixed culture were quite rhythmic in their rate of acid production; whereas strain P₂ was very slow. ML₈ and AM₂ were not uniform acid producers under the provided conditions, Figures 6 and 7. C₂, HP and the mixed strains showed very short lag phase prior to growth; whereas, AM₂, ML₈ and P₂ had longer lag phases than C₂, HP and the mixed strains. P₂ had the longest lag phase, Figures 8 and 9. For the production of proteolytic enzymes an almost similar pattern was observed for all the organisms, but as compared to Figures 6, 7, 8 and 9 there was a uniform increase in soluble nitrogen by AM₂, ML₈ and P₂ at a low level. It was also observed that during the stationary phase of growth, organisms produced maximum amounts of proteolytic enzymes, Figures 10 and 11. Figures 10 and 11 also show that strains C₂ HP, the mixed culture, AM₂, ML₈ and P₂ respectively produced proteolytic enzymes in a decreasing order. A non-sludge producing strain, S. lactis-C₂ which is most favorably used in Cottage cheese was selected for further study.
Figure 6. Rate of acid production by various strains of lactic streptococci in whey medium without regulation of pH.
Figure 7. Rate of acid production by various strains of lactic streptococci in NDM without regulation of pH.
Mixed culture
Strain c2
Strain Hp
Strain AM2
Strain ML8
Strain P2
Figure 8. Growth rate of various strains of lactic streptococci in whey medium. pH was regulated at 6.1 - 6.2.
Mixed culture
Strain C₂
Strain Hp
Strain AM₂
Strain ML₈
Strain P₂
Figure 9. Growth rate of various strains of lactic streptococci in NDM. pH was regulated at 6.1 - 6.2.
Figure 10. Representative curve for proteolytic activity of various strains of lactic streptococci in whey medium, at a controlled pH of 6.1 - 6.2.
Figure 11. Representative curve for proteolytic activity of various strains of lactic streptococci in NDM, at a controlled pH of 6.1 - 6.2.
Mixed culture
Strain C₂
Strain Hp
Strain AM₂
Strain ML₈
Strain P₂

Absorbancy at 274.5 nm

Incubation time (hours)

6 12 18 24 30 36
Purification of Proteolytic Enzymes

S. lactis-C<sub>2</sub> strain grown in a whey medium was used for enzyme production and purification. To ascertain whether the enzyme activity shown by the crude preparation was due to the presence of organisms or the presence of extracellular enzymes, the crude preparation was centrifuged for 30 min at 27000 G. Enzyme activity was determined in the cell free preparation. A summary of the purification at various steps is given in Table 1. A considerable drop in percent yield can be seen in Table 1. The third step of purification: DEAE Sephadex chromatography, served significantly better than the other steps in purification. The results of the DEAE Sephadex chromatography, in terms of the elution of total protein and the enzyme activity, are indicated in Figure 12. Two peaks of activity were noted.

Kinetic Studies

Enzyme preparation for kinetic study was prepared as stated earlier (Fraction III, p. 31) and as a substrate, 2.5% casein solution was used. Assay tubes showed optimum enzyme activity at 274.5 nm on DB-G spectrophotometer. The conditions were described in Materials and Methods. Each point on the curves represents an average measurement from three identical experiments in duplicate.
Table 1. Purification of proteolytic enzymes from *S. lactis*-C₂

<table>
<thead>
<tr>
<th>Stage and procedure</th>
<th>Volume (ml)</th>
<th>Total Protein (mg)</th>
<th>Purification (fold)</th>
<th>Yield (%)</th>
<th>Specific activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Crude</td>
<td>272.2</td>
<td>1125.000</td>
<td>1</td>
<td>100.000</td>
<td>34</td>
</tr>
<tr>
<td>2. Supernatant</td>
<td>250.0</td>
<td>250.000</td>
<td>4</td>
<td>22.222</td>
<td>205</td>
</tr>
<tr>
<td>3. Ammonium Sulfate</td>
<td>25.0</td>
<td>50.000</td>
<td>10</td>
<td>4.444</td>
<td>347</td>
</tr>
<tr>
<td>(40 - 75%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4. DEAE - Sephadex</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Peak I</td>
<td>50.0</td>
<td>1.310</td>
<td>1006</td>
<td>0.012</td>
<td>33720</td>
</tr>
<tr>
<td>Peak II</td>
<td>30.0</td>
<td>5.550</td>
<td>66</td>
<td>0.049</td>
<td>2472</td>
</tr>
</tbody>
</table>
Figure 12. Elution of protease enzymes from DEAE-Sephadex column. I and II represent the two major peaks of enzyme activity.
Absorbancy at 274.5 nm (enzyme)
The number of bacteria in the assay mixture was determined to be sure that the soluble nitrogen, measured at 274.5 nm was due to enzymatic activity and not due to growth of bacteria in the preparation during the 6 hr incubation period. There was no significant increase in the bacterial count indicating that enzymatic activity was responsible for the release of nonprotein nitrogen (NPN) in the assay mixture (Table 2). Increasing quantities of partially purified enzyme preparation (from Fraction III) were also incubated in a series of otherwise identical incubation mixtures. The release of NPN in each of these mixtures was proportional to the amount of enzyme present. This showed that the soluble nitrogen was being produced enzymatically.

**Effect of time on activity**

Identical mixtures containing a fixed level of enzyme and substrate were incubated for varying periods of time. For the first hour the initial rate of proteolysis was not significant, but between one hour and six hours the release of soluble nitrogen was proportional to the time of incubation. After 6 hr incubation at 32 C the amount of soluble nitrogen released leveled off. This may be due to the utilization of substrate present in the incubation mixture. Therefore all subsequent assays were carried out for only 5 hr.
Table 2. Cell count of *S. lactis*-C$_2$ on nutrient agar with 0.5% NDM during time of incubation of crude enzyme assay

<table>
<thead>
<tr>
<th>Time</th>
<th>No. of organisms/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>$53.6 \times 10^7$</td>
</tr>
<tr>
<td>15 min.</td>
<td>$43.5 \times 10^7$</td>
</tr>
<tr>
<td>30 min.</td>
<td>$42.1 \times 10^7$</td>
</tr>
<tr>
<td>1 hr.</td>
<td>$43.2 \times 10^7$</td>
</tr>
<tr>
<td>2 hr.</td>
<td>$43.0 \times 10^7$</td>
</tr>
<tr>
<td>3 hr.</td>
<td>$44.0 \times 10^7$</td>
</tr>
<tr>
<td>4 hr.</td>
<td>$40.5 \times 10^7$</td>
</tr>
<tr>
<td>5 hr.</td>
<td>$42.3 \times 10^7$</td>
</tr>
<tr>
<td>6 hr.</td>
<td>$41.4 \times 10^7$</td>
</tr>
</tbody>
</table>
Figure 13. Influence of enzyme concentration on rate of non-protein nitrogen (NPN) release.
Absorbancy at 274.5 nm

Enzyme concentration (gamma protein/assay mixture)
Figure 14. Effect of time on enzyme activity as measured by absorbency.
Absorbancy at 274.5 nm vs. Time (hours)
Effect of substrate concentration on activity

A constant amount of enzyme preparation with increasing concentrations of casein solution was used to test the effects of substrate concentration. Saturation of enzyme was obtained with 7% substrate for this enzyme concentration. A linear relationship was observed with added levels of substrate up to 7% casein per assay tube, Figure 15.

Effect of pH on activity

Effects of pH on specific activity was determined by varying the pH of the casein substrate solution (range 5.5 to 12.00 pH). Maximum activity was observed at pH 6.2, Figure 16.

Effect of temperature on activity

It is difficult to say that a particular temperature is optimum for enzyme activity because with an increase of every 10°C enzyme activity increases two-fold. For this reason no particular optimum temperature study was carried out. But on a temperature bar enzyme activity was assayed between the temperature range of 20 - 55°C. A linear increase in activity was observed between 25°C and 50°C after incubation at pH 6.2, Figure 17.
Figure 15. Influence of substrate concentration on enzyme activity as measured by absorbancy.
Absorbancy at 274.5 nm

Substrate concentration (%)

Graph showing the absorbancy at 274.5 nm as a function of substrate concentration (%).
Figure 16. Effect of pH on enzyme activity as measured by absorbancy.
Absorbancy at 274.5 nm
Figure 17. Effect of incubation temperature on enzyme activity.
Stability of the enzyme

No systematic studies on the stability of the proteolytic enzymes were carried out at low temperature, but it was observed that crude, crude freeze dried and highly purified enzyme preparations lost only negligible activity after storage at -20°C for 14-16 weeks. Repeated freezing at -20°C and thawing at room temperature had no significant effect on enzyme activity.

At 80°C cell-free extract was tested for heat stability of enzyme. Twenty-five ml of the enzyme (Fraction III) in a 100 ml Erlenmeyer flask was held in an 80°C water bath. At 5 min intervals, 0.5 samples were removed and immediately placed sequentially in 10 ml of 2.5%, pH 6.2 casein solution. Enzyme assay was carried out as previously indicated. Initially a slight increase in activity was noted and after 20 min activity gradually declined, until at the end of 80 min 41% loss in activity had occurred as shown in Figure 18.

Effect of Preculturing on Direct Acid Cottage Cheese

Prior to the acidification of skimmilk to produce direct acid Cottage cheese, addition of starter had a significant effect on body and texture of the cheese (Tables 3 and 4). Average firmness and moisture content of the un-creamed curd also increased significantly, Figures 19 and 20. Brackets at the top of each bar represent the standard deviations. These results show that the length of growth of organisms in the milk has a noticeable affect on the quality of the curd produced.
Figure 18. Effect of heating on enzyme activity. Each incubation mixture contained 10 mg protein of Fraction III and 10 ml of 2.5% casein solution (pH 6.2). Incubation mixture was heated at 80°C.
Table 3. Effect of partial culturing on average body and texture scores of creamed direct acid Cottage cheese (1. superior, 2. excellent, 3. satisfactory, 4. objectionable, 5. unsaleable).

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Direct acid</th>
<th>Cultured to pH 6.0</th>
<th>Cultured to pH 5.5</th>
<th>Culture control</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Direct acid</td>
<td>3.70*</td>
<td>3.11*</td>
<td>2.88</td>
</tr>
<tr>
<td>2.</td>
<td>Cultured</td>
<td>3.11*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3.</td>
<td>Cultured</td>
<td></td>
<td>2.80**</td>
<td></td>
</tr>
<tr>
<td>4.</td>
<td>Culture</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Difference between treatment and control is significant at 0.01 level
**Difference between treatment and control is not significant at 0.01 level.

Table 4. Analysis of variance of the average body and texture scores of creamed direct acid Cottage cheese

<table>
<thead>
<tr>
<th>Source</th>
<th>df</th>
<th>SS</th>
<th>MS</th>
<th>F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Replicates</td>
<td>9(r-1)</td>
<td>2.65</td>
<td>0.294</td>
<td>1.26</td>
</tr>
<tr>
<td>Treatments</td>
<td>3(t-1)</td>
<td>25.12</td>
<td>8.37</td>
<td>*35.62</td>
</tr>
<tr>
<td>Judges</td>
<td>4(j-1)</td>
<td>6.60</td>
<td>1.65</td>
<td>*7.02</td>
</tr>
<tr>
<td>Judges x Treat.</td>
<td>12(j-1)(t-1)</td>
<td>13.88</td>
<td>1.16</td>
<td>4.94</td>
</tr>
<tr>
<td>Error</td>
<td>171(r-1)(tj-1)</td>
<td>40.13</td>
<td>0.235</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>199(rtj-1)</td>
<td>88.38</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Significant at 0.01 level.
Figure 19. Effect of partial culturing on the firmness of uncreamed direct acid Cottage cheese. 1- Direct acid, 2- Cultured to pH 6.0 prior to direct acidification, 3- Cultured to pH 5.5 prior to direct acidification, 4- Culture control.
Figure 20. Effect of partial culturing on the moisture content of uncreamed direct acid Cottage cheese. 1- Direct acid, 2- Cultured to pH 6.0 prior to direct acidification, 3- Cultured pH 5.5 prior to direct acidification, 4- Culture control.
A minimum of 40-45 replications of each treatment were carried out but for the final judgment 10 batches of cheese for each treatment were made in a one week period.

**Effect of Proteolytic Enzymes on Direct Acid Cottage Cheese**

In contrast to preculturing, a small amount of freeze dried crude enzyme preparation was added for a shorter period of time. Addition, prior to acidification of skimmilk, has a significant effect on the body and texture of the final product. Analysis of variance of the sensory score and average grading of creamed Cottage cheese also agree (Tables 5 and 6) as indicated by a marked improvement in firmness and moisture retaining capacity of the curd, Figures 21 and 22. Slightly higher firmness was measured on curd, prepared with 1% enzyme preparation. This curd was also judged as very tough, dry and mealy. A minimum of 40-45 replications of each treatment were carried out, but for the final judgment eight batches of cheese for each treatment were made in a one week period.

**Continuous Direct Acid Cottage Cheese Making of Partially Cultured Skimmilk**

Direct acid Cottage cheese was made using partially cultured skimmilk (pH 5.5). Five judges graded the cheese and gave 2.25 average grade to
cheese made from partially cultured skimmilk acidified to 4.67 using cold concentrated HCl; in contrast to this, direct acid Cottage cheese was graded as 3.75.
Table 5. Effect of freeze dried crude enzyme obtained from *S. lactis*-C₂ on average body and texture scores of creamed direct acid Cottage cheese. (1. superior, 2. excellent, 3. satisfactory, 4. objectionable, 5. unsaleable.)

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Direct acid only</th>
<th>1% crude enzyme + direct acid</th>
<th>0.5% crude enzyme + direct acid</th>
<th>Culture control</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3.67*</td>
<td>2.81*</td>
<td>2.52**</td>
<td>2.37</td>
<td></td>
</tr>
</tbody>
</table>

*Difference between treatment and control significant at 0.01 level.
**Difference between treatment and control not significant at 0.01 level.
Tukey’s test for multiple mean comparison was used at α = 0.01
Q = 4.5 for 120 d.f.
   4.4 for ∞ d.f.

Table 6. Analysis of variance of the average body and texture scores of creamed direct acid Cottage cheese

<table>
<thead>
<tr>
<th>Source</th>
<th>df</th>
<th>SS</th>
<th>MS</th>
<th>F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Replicates</td>
<td>7(r-1)</td>
<td>2.925</td>
<td>0.4179</td>
<td>1.15</td>
</tr>
<tr>
<td>Treatments</td>
<td>3(t-1)</td>
<td>48.394</td>
<td>16.1333</td>
<td>44.5</td>
</tr>
<tr>
<td>Judges</td>
<td>5(j-1)</td>
<td>3.050</td>
<td>0.6100</td>
<td>1.6</td>
</tr>
<tr>
<td>Judges x Treat.</td>
<td>15(j-1)(t-1)</td>
<td>4.856</td>
<td>0.3237</td>
<td></td>
</tr>
<tr>
<td>Error</td>
<td>161(r-1)(tj-1)</td>
<td>58.325</td>
<td>0.3622</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>191(rtj-1)</td>
<td>117.55</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Difference significant at 0.01 level.
Figure 21. Effect of freeze dried crude enzyme preparation on the firmness of uncreamed direct acid Cottage cheese. 1- Direct acid, 2- 1% crude enzyme + direct acid, 3- 0.5% crude enzyme + direct acid, 4- Culture control.
Figure 22. Effect of freeze dried crude enzyme preparation on the moisture content of uncreamed direct acid Cottage cheese. 1- Direct acid, 2- 1% crude enzyme + direct acid, 3- 0.5% crude enzyme + direct acid, 4- Culture control.
DISCUSSION

Limited incubation of skimmilk containing 5% starter improved the body and texture of the direct acid Cottage cheese curd. Similar improvement was achieved by incubating the skimmilk with a freeze-dried crude culture concentrate for a short time (1 hr) during which no growth of organisms was observed. The improvements in curd quality were attributed to factors in the crude concentrate produced by the organisms. Partial preculturing was successfully used in the continuous process for direct acid Cottage cheese making. However, it was necessary to cook the curd to 63.6 °C to obtain good bodied cheese. Best results were obtained when partially cultured skimmilk was acidified to pH 4.67 in comparison to noncultured controls where milk was acidified to pH 4.60. This might have contributed to the need for the higher cooking temperature. Addition of rennet, prior to acidification in this continuous process had adverse effects: at lower pH (4.6) milk was partly coagulated before it entered the curd-former and at higher pH values (4.7 or above) it caused a tough, rubbery curd.

Proteolytic activity of starter bacteria was considered by some workers to contribute significantly to the firmness of Cottage cheese curd (57, 62, 122); whereas others have considered that pH is mainly responsible for the firmness of curd and proteolysis is of minor importance (39, 42, 58). Since NPN
increases during Cottage cheese setting (62), proteolytic enzymes produced by the organisms (S. lactis - C2) during growth could be responsible for the improved body and texture of the Cottage cheese. This assumption was not positively established in this study. It could be demonstrated only by making good quality direct acid Cottage cheese using highly purified proteolytic enzymes isolated from the starter organisms.

A 1000-fold purification (protein basis) of extra-cellular proteolytic activity produced by S. lactis-C2 was achieved as compared to 152-fold purification of intracellular protease from a mutant of S. lactis by Westhoff, et al. (121), and 120-fold purification of extracellular protease from S. lactis by Williamson et al. (123). The purified preparation was most active on casein at pH 6.2. In S. lactis (pH 7.0), Williamson et al. (123) reported that purified extracellular enzymes from S. lactis were similar to trypsin having an alkaline pH optimum (pH 8.5) and remarkable heat stability. The protease of S. lactis-C2 was also highly heat stable but in contrast to Williamson et al. (123) activity optimum occurred at pH 6.2. Several strains of lactic streptococci grown in the reconstituted NDM and whey medium varied in growth rate, rate of acid production and rate of enzyme production. These studies were in agreement with other researchers who have suggested lactic culture variability (13, 53).

Production of proteases by S. lactis in the initial stages of growth have been reported (114, 122, 124) but under conditions of this study all organisms produced maximum activity during their stationary phase of growth. This
discrepancy may be due to the use of pure culture in the present study as compared to a mixed culture used by the previous workers. Fortification of growth media with simple nitrogen compounds stimulated the organisms to increased proteolytic enzyme production (1, 18, 66, 79, 115, 118), which also held true in the present investigation where addition of Lactivate powder to the whey medium stimulated the organisms. But in contrast to this Speck (101) reported the sharp reduction in proteolysis when milk was supplemented with pancreas extract.

Three possibilities for continuous direct acid Cottage cheese making have emerged from this investigation.

1. **Batch process**: Skimmilk can be partially fermented in a vat prior to acidification at 4°C. Direct acid Cottage cheese can then be made in a curd former as described by Ernstrom (44).

2. **Crude culture concentrate**: Skimmilk can be incubated for short periods with culture concentrate prior to acidification. Direct acid Cottage cheese can be made in the curd former.

3. **Continuous fermentation**: Starter organisms may be grown under controlled conditions to produce maximum proteolytic enzymes using the chemostat principle. Continuous addition of skimmilk to the fermenter will commence with the removal of fermented skimmilk.

The principle objectives in direct acid Cottage cheese making were to avoid the problems associated with starter organisms in the conventional procedures and a saving in labor and time.
The batch method can save time (5 hr of incubation for conventional process to 2.5 hr for batch process) and is adaptable for continuous Cottage cheese making but problems associated with starter organisms are still unavoidable. On the other hand, after the incubation of skimmilk with crude culture concentrate for one hr, direct acid Cottage cheese can be made by the continuous process. This can save more time than the batch process. Important factors which can prevent the wide use of this process include dilution of cheese milk if liquid concentrate is used and cost of making the freeze-dried powder of crude culture concentrate if powder is used. Another possibility is continuous fermentation where organisms can be grown under optimum conditions for the production of proteolytic enzymes. This preparation can be used continuously in continuous direct acid Cottage cheese making. The most important factor that can prevent its practical use is the problem of bacteriophage.

Lloyd (72) reviewed several possibilities of continuous fermentation, but so far most of the workers were concerned about the concentration of biomass or number of organisms and their ability to produce acid. The idea to concentrate the factors involved in improving the body and texture of Cottage cheese rather than concentration of the organisms themselves emerged for the first time from the present investigation.

This work also opened the door to the successful use of direct acidification technique in other varieties of cheese, e.g. Cheddar, Monterey, which could bring revolutionary changes to the cheese industry. This would allow industries to switch from conventional batch processes to more economical and
efficient continuous direct acid processes. Cheese varieties requiring ripening are usually considered difficult to make by direct acidification processes. Direct acidification eliminates the organisms required for ripening, but use of a culture concentrate may solve the problem and could lead to continuous cheese making for other varieties of cheese.
CONCLUSIONS

1. Proteolytic activity of all the lactic streptococci organisms tested was maximum during their stationary phase of growth.

2. In the whey medium, Lactivate powder had stimulatory effects on growth rate, rate of acid production and production of proteolytic enzyme.

3. Partial culturing of skimmilk prior to acidification for Cottage cheese makes significantly improved the body and texture of Cottage cheese curd.

4. Incubation of skimmilk containing freeze dried crude enzyme preparation prior to acidification also improved the body and texture of Cottage cheese.

5. In addition to acid production, microorganisms played an important role in improving the quality of direct acid Cottage cheese curd. This may be attributed to their proteolytic activity in the milk.

6. Partial culturing of skimmilk prior to acidification was successfully used in the continuous Cottage cheese process and provided good quality curd.

7. The proteolytic enzyme *S. lactis*-C₂ was purified 1000 fold.

8. The enzyme was extracellular and had a pH optimum of 6.2 at 32 C.

9. Enzyme was active between 25 to 50 C for 5 hr incubation at pH 6.2 and it was stable for 20 min at 80 C while a 41% loss in activity was observed in 80 min.
BIBLIOGRAPHY


VITA

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Doctor of Philosophy

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