SCREENING FOR COAGULATING ENZYMES FROM BACTERIAL SOURCES

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INTRODUCTION

Milk-clotting enzymes have been used in the manufacture of cheese and similar foods since antiquity.(89). These enzymes are as diverse in their individual properties and specificities as the sources from which they come. Coagulation of the casein micelle in milk is brought about by proteolytic enzymes obtained from plant, animal, and microbial sources. In modern commercial cheesemaking, the animal enzyme, rennin, is used. Rennin is an enzyme present in the abomasum of the suckling calf. The term rennet or rennet extract is used to denote the water and sodium chloride extract of rennin. Commercial rennet extract is used in the manufacture of a wide variety of cheeses.

It was not until 1815 - 1850 when rennet as we know it today was extracted from the abomasum of the suckling bovine calf. This has provided the best and most reliable source of rennet extract to date. A reason for its widespread use is that calves are the largest and most commonly available animal as a source of rennet, and provide the greatest material yield per animal. Another advantage for the use of calf rennet is the high degree of specificity of rennet action on bovine milk. Bovine milk is the most widely used in cheese manufacturing. The use of calf rennet as a coagulating agent, therefore, has long been commonplace. It has commanded a major interest in the dairy industry because of its widespread use in cheese manufacturing.

It is, however, with discerning interest that investigators are now looking to different sources for rennet-like enzymes. In recent years, the number of young calves slaughtered (the principle source of rennet extract) has decreased 40%. At the same time, the cheese industry has expanded its

production by 25% (66). The relatively high cost of calf rennet coupled with considerable price fluctuation and variation in supply has made it imperative to search for alternative sources of rennet-like enzymes. The highly competitive nature of today's consumer markets and the relative importance of cheese in the diet further warrants search for cheaper sources of rennet supply.

Furthermore, in many areas of the world, per capita consumption of cheese and other coagulated dairy products could be enhanced if religious and dietary restrictions were lessened. Hence, it would be desirable to have rennet-like enzymes of nonanimal origin.

For the foregoing reasons, the study reported in this thesis was undertaken. The objectives of the work were to: (a) screen possible bacterial sources for rennet-like enzymes, (b) study and evaluate the enzymes elaborated, and (c) select one bacterial culture for quantitative production and concentration of the rennet-like enzymes.

LITERATURE REVIEW

It is not known exactly when or where the use of rennet as a milk coagulant began. Indeed, it is thought the discovery of rennet was quite by accident, and resulted from the use of excised animal stomachs as storage and transport containers for milk. It is supposed that this practice first occurred somewhere in Asia Minor. History records the Assyrians, Babylonians, Greeks, and Romans making extensive use of coagulated dairy products and developing some of the early art of cheesemaking. As early as 349-249 B.C. the Greeks obtained a milk coagulant from the fig tree. The practice carried

over and was used by the Romans, where cheese became an increasing mainstay in the diet. In addition to rennet obtained from rabbits, goats, and sheep, the Romans used milk coagulants from the flower of field thistles, seeds of safflower, and green bark of the fig tree. Since that early time, coagulated dairy products have provided man with an efficient means of food preservation, thus contributing to his nutrition and welfare (52).

Properties and Action of Calf Rennet

In searching for a substitute for calf rennet, some understanding of rennet characteristics and action is desirable. Extensive research has involved calf rennet. Initially, work revolved around improved methods of extraction, purification, stablization, preservation of activity, and standardization. Placek et al (62), in a collaborative report, outlined the commercial preparation of rennet extract. The enzyme is prepared by a salting-out procedure and activated with 18% HCl.

Several workers (16, 17, 19, 37) have done extensive work on the crystallization of pure rennin. Foltman (30) prepared crystalline rennin in the form of rectangular blocks and plates. Crystalline rennin is considered not to be homogeneous. Foltman (31) obtained three active fractions when rennin was fractionated using DEAE cellulose column chromatography. Ernstrom (29) observed four electrophoretic components of rennin in 0.033 M phosphate buffer, pH 6.8. Purified rennin has been found to be a typical globulin protein of molecular weight 34,000 (43).

Mickelsen and Ernstom (56) established that rennet is most stable between pH 5.0 and 6.0. Above pH 6.0, activity losses increased with pH and

were highly temperature dependent. A region of instability also was noted between pH 3.0 to 4.9. It was implied that activity losses were due in part to autolysis (30).

There is still no agreement among researchers as to the specific action of rennet on milk. Milk clotting is believed to be a complex process involving both enzymic and nonenzymic reactions (89). Lindqvist (49), in his review, described the action of rennet on casein occurring in three phases. The primary phase involved breakdown of α-casein. Also during the primary phase, κ-casein, a protective colloid of α-casein, was split into two fractions (14). The soluble fraction contained a high molecular weight glycomacropeptide (4). The secondary phase occurred simultaneously with the primary phase and involved conversion of the caseins to paracaseins which were susceptible to action of ionic calcium. Calcium ions reacted with the paracasein to form complexes or calcium bridges which resulted in the clotting of milk by precipitation (39). The tertiary phase of rennet action was a nonspecific proteolysis of the casein fraction occurring during the ripening period (49).

Certain casein fractions are more sensitive to rennet action than are others. Lahav and Babad (46) concluded that only α -casein fraction was altered by rennet; whereas, β and γ-casein were not attacked but merely co-precipitated unchanged along with the altered α-casein. Ledford et al (47, 48) showed electrophoretically that β-casein in laboratory manufactured cheese remained unchanged even on extended periods of storage. Using starch gel electrophoresis, El-Negoumy (26) showed that of the whole casein

fraction only the κ-casein component was altered by rennet in the 70 minute period studied. Indeed, the κ-casein band had mostly disappeared after five minutes of rennet action. The β-casein for the most part remained unchanged.

Other Milk-Clotting Enzymes from Animals

All proteolytic enzymes will coagulate milk under proper conditions. In seeking rennet substitutes, therefore, investigators have studied other proteolytic animal enzymes. Among these, pepsin, trypsin, and chymotrypsin have been considered. Trypsin produces only a weak clot in milk due to extensive protein digestion. Trypsin often proteolyzes the casein so extensively that no clot can form (89). Chymotrypsin is another animal protease of pancreatic origin which possesses milk-clotting activity. However, it has found only limited use due to its proteolytic action on β-casein and the formation of bitter peptides in cheese.

Pepsin is very much like rennet in its milk-clotting properties. It replaces rennet in the stomach as the animal matures, and is often found as a contaminant in commercial rennet extract. Pepsin first was used during World War I when there was a severe shortage of rennet (86). While favorable results were obtained in several trials using pepsin, the Research Committee of the National Cheese Institute stated in 1960 that pepsin should not be used as a complete substitute for rennet (55). This was partly a result of reports stating that pepsin produced bitterness in cheese. Since that time, however, pepsin has been used increasingly, both alone and in combination with rennet, in the successful manufacture of cheese (28).

Maragoudakis et al (51) found that cheese made with pepsin evidenced no bitter flavor and, in fact, had a better flavor after 150 days of storage than rennet cheese. Others have shown that nonprotein nitrogen (NPN) and non-casein nitrogen (NCN) production during cheese ripening was greater for rennet cheese than for pepsin cheese (53). Studies have shown that pepsin is more susceptible to change in pH in the range of 6.5 - 6.8 (in terms of activity loss) when compared to animal rennet (28, 83). Aside from dietary and religious objections to animal source materials, pepsin is probably the most reliable rennet substitute of animal origin.

Milk-Clotting Enzymes from Plants

Milk-clotting enzymes of plant origin are important from the standpoint of alleviating economic problems of supply and demand, and negating religious and dietary objections. Vegetable proteases studied for rennet-like properties have been obtained from the withania bush berries of India, papaya tree, fig tree, pineapple, pumpkin, cocoa bean, and cynara petals (89). In most cases, all have good milk-clotting activity, and when used in optimum concentrations produced good cheeses (90). Papain, however, is notorious for its highly proteolytic activity which leads to bitter cheese. Of the vegetable rennet substitutes, ficin has found the widest use (45), while the enzyme from pumpkin latex most closely resembled the action of calf rennet (64). Generally, vegetable proteases show a wider range of temperature activity levels, higher optimum temperature, and higher heat destruction than that of rennet. In addition, pH optima are higher than rennet; and a longer time is necessary for coagulation to occur. Sasaki

et al (70) found that in general, vegetable rennets caused greatest decomposition of the casein, forming a softer curd when compared to calf rennet. Oosthuizen and Blair (60) noted that vegetable rennets attack an abnormally large part of the casein in a nonspecific proteolysis. Ganguli and Bhalerao (33, 34) demonstrated the action of vegetable rennet using casein-agar gels. The formation of one precipitate zone followed by a clear zone was different from the action of animal rennet, in which there were two precipitate zones followed by a clear zone.

Milk-Clotting Enzymes from Microorganisms

Probably the greatest potential source of rennet substitutes is microorganisms. Many bacteria secrete enzymes capable of clotting milk without producing enough acid to cause coagulation. Hundreds of species of microorganisms have been studied for their milk-clotting activity, and yet there are thousands more to be tested. The possibility of microbial enzymes proving successful as rennet substitutes is seen from the fact that microorganisms play an important role in the manufacture and ripening of cheese. Microbial enzymes exhibit considerable variation in the range of activity, substrate specificity, and mode of action. They also can be produced economically on any scale (9).

Microbial enzyme preparations exhibiting milk-clotting activity invariably are accompanied by proteolytic enzymes. Coagulation of milk with such enzyme preparations parallels the action of calf rennet, but the exact mechanism is not known. However, the nonspecific proteolysis of most microbial enzymes provides the basic difference in microbial and calf rennet.

Even with this serious handicap, the potential for microbial enzymes as rennet substitutes elaborated in a fermentative process is virtually limitless.

Fungal milk-clotting enzymes. Among the fungi found to produce rennet-like enzymes are the genera Aspergillus, Mucor, Endothia, Byssochlamys, Rhizopus, Ascochyta, Sclerotium, Collectotrichum, Phymatitrichum, and Penicillium.

Veringa (86) in 1961 reported from the literature on the scope of research being conducted on rennet substitutes. The author reported that enzyme preparations of several fungi had been used with varying degrees of success in the manufacture of hard cheeses. Meitro Sangy Kabushiki Kaisha, Naogya, Japan produced a fungal rennet patented by Arima and Iwasaki (4, 5). The milk-clotting enzyme was produced from Mucor hemaris, cultured by a wheat bran koji process. Tsugo (85) isolated an enzyme from Mucor Pusillus Uar Lindt which had 20 - 25% more milk-clotting activity than calf rennet. From nearly 800 strains of micro organisms screened for milk-clotting activity, Arima et al (6) selected the same organism because its enzymic milk-clotting and proteolytic activity resembled that of calf rennet. Poleva and Popova (61) investigated six species of Asperigillus fungi grown on various media and found A.parasiticus to have the highest milk-clotting activity when grown in maize-casein medium. Albonico et al (2) isolated a comparable enzyme from A. orazyae. Rotini (68) compared the milk-coagulating enzyme of A. orazyae with 8 other rennets of human, animal, plant, and bacterial sources.

Knight (44) surveyed 39 molds for the ability to produce rennet-like enzymes. Out of these, Byssochlamys fulva produced the most consistent results and highest milk-clotting activity. It was selected for further study. Srinivasan et al (77) screened 230 cultures of fungi for milk-clotting activity. The isolates were divided into three groups of high, medium, and low milk-clotting activity. The high activity group invariably yielded loose textured curd when cheese was made, attributable to the high proteolytic activity of the molds. Wang et al (88) studied 44 strains of four species of Rhizopus for their ability to produce milk-clotting enzymes. One of the organisms, R. oligosporus, was selected for further investigation. In other work, Sardinas (69) examined a rennet enzyme of Endothia parasitica. Of some 540 fungi tested, only this organism gave favorable results as a rennet substitute. The rennet enzyme of E. parasitica is now being marketed in the United States. Limited commercial use also is being made of the milk coagulant of Mucor pusillus var. lindt in this country, and to a greater extent in New Zealand, Australia, and Japan (68).

Bacterial milk-clotting enzymes. Among the bacterial genera that produce rennet-like enzymes, the spore forming Bacillus, and Pseudomonas, Serratia, Aeromonas, Actinomycetes, and Streptomycetes have received the most attention. In addition, many other bacteria have been screened for their milk-clotting ability. As early as 1928, Whalin (87) did extensive work with Bacillus prodigiosus in the development of a rennet-like enzyme. Shimwell and Evans (71) made satisfactory Kosher cheese from enzymes of several species of Bacillus. Barkan et al (11) found the enzyme from B. mesentericus to be 50% more active than calf rennet. Other workers have

done extensive work in isolating and purifying milk-clotting enzymes from several species of Bacillus (20, 81). Srinivsan et al (77) screened some 43 cultures of bacteria for milk-clotting activity isolated from such sources as soil, butter, and milk. In preliminary work, Sardinas (69) screened some 380 bacteria and found none to be suitable as a rennet substitute.

Screening Microorganisms for Milk-Clotting Enzymes

The great number of potential microorganisms as sources of rennet substitutes has presented some problem in the screening of milk-clotting enzymes. There have been a number of methods used; and they generally rely on the criteria of simplicity, materials available, and the class of organisms being studied. More elaborate methods have been used, however, that allude not only to the enzyme's ability to clot milk, but also to other general characteristics of enzymes produced by a specific organism. In most cases, the microorganisms isolated from various sources, have been grown in a suitable growth medium under a number of fermentative processes for maximum enzyme production. The elaborated enzyme then has been tested for milk-clotting activity directly from the growth medium, or has been purified further or concentrated by various chemical and biochemical procedures before testing the activity. To isolate microbial rennets, the cell free extracts from the fermentation media are used and purified by dialysis, precipitation with salt, or fractionation with ethyl alcohol or acetone. The enzyme preparations are usually dried or stored at low temperatures for preservation.

Media. At present, it appears that the best milk-clotting enzyme production is obtained on milk or peptone medium with wheat bran and calcium phytate added, and grown under conditions of high aeration and agitation. Initial work conducted by Wahlin (87) showed that rennet production by B. prodigiosus occurred best in a medium containing complex proteins; whereas rennet production was low in synthetic medium consisting of proteose-peptones and free amino acids. He concluded that a complex protein medium containing certain peptide bonds was required to effect enzyme elaboration. Emaniuloff (27) used a medium consisting of wheat bran, oats, potatoes, barley, and water to produce a protease enzyme. The use of milk as a growth medium yielded very low quantities of milk-clotting enzyme when B. cereus and B. subtilis were studied (75). However, skimmilk digested with papain proved to be quite effective as a growth medium (76). In other studies, Babbar et al (8) cultivated fungi on semisolid wheat bran medium and bacteria in autoclaved skimmilk. The bacteria were grown for 72 hours under submerged and surface conditions at 30 C. Submerged conditions usually involve the use of a rotary shaker, which also provides a means of aeration and agitation.

Natural media have been found to be more suitable for milk-clotting enzyme elaboration from microorganisms than synthetic media. The stimulation of enzyme production in natural media has been attributed to the presence of an abundant supply of proteins and carbohydrates, along with some of the undefined growth factors such as vitamins and calcium phytate (9).

The effect of calcium phytate on the production of milk-clotting and proteolytic enzymes of certain bacteria was reported by Dudani (24).

Knight (44), in screening mold isolates, grew the organisms at 25 C on a rotary shaker in undiluted whey or 2% cornsteep liquor plus 2% lactose. Samples were removed periodically and assayed for milk-clotting activity. Arima et al (6) used a semisynthetic media in which the cultures were incubated at 30 C up to 10 days. After cultivation, the culture medium was centrifuged and the supernatant used for enzyme assay. Sardinas (69) employed the use of a soybean meal supplemented with cerelose and skimmilk. The supernatant broth was assayed for milk-clotting and total proteolytic activity.

In addition to the type of media employed for enzyme elaboration, attention also must be given to such factors as pH of the media, temperature and period of incubation, and the mode of growth of the microorganisms (12).

Determination of milk-clotting activity. A number of methods for assaying enzyme activity have been reported. All are basically similar and differ only in the type and amount of substrate used, and the method of assigning activity. In most of these methods, the activity of the milk-clotting enzyme is determined by measuring the time required to clot a suitable substrate (fresh milk or skimmilk powder) supplemented with varying levels of calcium chloride and under defined conditions of temperature.

In most cases, the enzyme concentration is so chosen that the clotting time is inversely proportional to the enzyme activity (83). One unit of activity is defined as the amount of enzyme that will clot 1 ml of substrate in 1 minute at a specified temperature (18).

Srinivasan et al (77) reported milk-clotting activity as represented by the formula 60/t X DF, where "t" is the time in seconds for clotting to occur and "DF" is the dilution factor. Under these conditions, the time of clotting was found to be inversely proportional to the enzyme activity except for higher than five-fold dilutions.

Determination of proteolytic activity. Because of the inherent hydrolytic nature of most microbial enzymes, it also has been necessary to determine the total proteolytic activity. It is believed that enzymes responsible for milk clotting and proteolysis are two separate and independent moieties (24). Therefore, a separate test has been employed for assay of proteolytic activity. Some workers have gone so far as to establish a ratio of milk-clotting activity to proteolytic activity, referred to as an efficiency index for rapid screening of microorganisms. Enzymes exhibiting indexes near the value for calf rennet were considered to be possible sources of suitable enzyme (24). Generally, the method used to measure proteolytic activity has been the Folin-Ciocaltau determination of soluble tyrosine-tryptophan content of enzyme hydrolyzed casein (40). Modifications of this procedure also have been employed, expressing enzyme activity as optical density (22). Richardson et al (66) and Puhan and Steffen (63) assessed the proteolytic activity by measuring the NPN increase during incubation in a casein substrate. Melachouris and Tuckey (54) measured NPN released when casein fractions were used as a substrate.

In other screening tests, workers have used the casein-agar gel plate technique (12, 65). Casein dispersed in agar gave characteristic patterns of precipitation and proteolysis when incubated with different enzyme

preparations. In addition, electrophoresis studies of enzyme-treated milk and casein substrates have given qualitative information on the proteolytic nature of microbial enzymes, as well as their suitability as a rennet substitute (84).

Microbial Enzyme Isolation

In most of the screening tests, the crude enzyme preparation as elaborated by the microorganism in a suitable substrate is checked for its rennet-like properties. However, in order to evaluate thoroughly a potential rennet substitute, it has been necessary to extract and isolate the enzyme in a semipurified or purified form. Usually protein precipitants such as ammonium sulfate or organic solvents--ethyl alcohol, acetone--have been employed at low temperatures. The precipitated enzyme then has been lyophilized or dried in-vacuo at low temperatures to avoid activity loss. Some workers have used methods of concentrating under vacuum--flash evaporation (69) and gel filtration (36) in attempts to purify microbial enzymes. Srinivasan (74) found that using ammonium sulfate as the protein precipitant gave the best results, and obtained an enzyme capable of coagulating 800 times its own weight of milk. Generally, the active protein fractions were obtained between 30 - 60% saturation with ammonium sulfate (4, 8, 44). Sardinas (69) and Arima and Iwasaki (5) patented the extraction of milk-clotting enzymes of E. parasitica and Mucor pusillus respectively by means of ammonium sulfate precipitation. In other work, Arima et al (7) purified Mucor rennet by column chromatography on Amberlite and Sephadex. The enzyme was further crystallized from solution by 40-50% saturation with ammonium sulfate.

Hagemeyer et al (36), in a series of 12 purification steps, used a combination of ammonium sulfate precipitation, Sephadex filtration, and DEAE cellulose column chromatography to purify a commercial preparation of the enzyme of E. parasitica. The same workers also used a combination of ammonium sulfate and organic solvent precipitation, charcoal treatment, gel filtration, and column chromatography to obtain a crystalline enzyme.

In all extraction procedures, the milk-clotting activity is checked at various intervals and, in most cases, the protein content of each fraction is assayed for specific activity determination.

Microbial Milk-Clotting Enzyme Characteristics

Effect of temperature. Generally the rennet substitutes which have been used in cheesemaking trials have higher optimal temperatures than calf rennet. Most microbial enzymes, however, are relatively thermolable. The maximum speed of clotting was obtained at 75 - 80 C for a microbial rennet from B. cereus (54). The milk-clotting enzyme of B. subtitis was completely inactivated at 50 C for 15 minutes (8). Milk-clotting enzymes of Fomitopsis pinicola and A. usameii had temperature optima of 48 C and 30 - 52 C respectively (58, 32). Tsugo et al (83) found that most microbial rennets had optimal temperatures over 60 C for 5 minutes (69). The enzyme of Mucor pusillus was more heat resistant and had a lower temperature optimum than calf rennet (41, 66).

Effect of pH. The milk-clotting activity of microbial rennets is generally less affected by changes in pH than is calf rennet, and the optima vary considerably from enzyme to enzyme. Indeed, the optimum for

E. parasitica is between pH 4.0 - 5.0 (36); whereas, for B. cereus the optimum lies near pH 7.0 (54). Yu et al (91) found the optimum pH for milk-clotting for Mucor pusillus var. Lindt to be 5.5. The enzyme was most stable at pH 5.0. Babel and Somkuti (10) found for the Mucor enzyme, coagulation time was reduced by 50% when the substrate pH was lowered from 6.6 to 6.0 using lactic acid, and by 82% when HCl was used. The enzyme of F. pinicola was more sensitive to pH than calf rennet, with the optimum at pH 2.6 (58).

Effect of calcium ion concentration. Microbial rennets are much more affected by calcium ion concentration than is calf rennet. Alais and Novak (1), however, found the enzyme of E. parasitica was less sensitive to changes in calcium ion concentration. Fox (32) also found A. usameii rennet to be dependent on calcium ion concentration. In general increased calcium ion concentration tends to increase milk-clotting activity to a point at which further addition may actually decrease activity. In some cases, it was necessary to add varying amounts of calcium chloride to the cheese milk to insure proper coagulation and normal curd properties, when a given strength of microbial rennet was used(85).

Proteolytic properties. Probably the most distinguishing characteristic of most microbial rennets is the nonspecific type of proteolytic activity. It is this particular property that has presented the greatest problem in producing a suitable rennet substitute of microbial origin. Depending on the source of the enzyme and substrate conditions of pH, temperature, and calcium ion concentration, varying degrees of proteolysis have been observed. It seems, however, that there are optimum conditions

at which the proteolysis by the enzymes is kept to a minimum (50). Reports vary on the exact mode of action of these proteolytic enzymes and on the casein fraction being attacked. The enzymes of B. cereus preferentially hydrolyzed the β-casein fraction (54); whereas, the Mucor pusillus enzymes attacked α_S-casein as well as κ-casein (41). Other enzymes produced marked changes in the β-casein fraction which led to an increased content of NPN and the production of bitter peptides in the cheese (84). Electrophoresis examination of results of enzymic action on milk showed that microbial enzymes with strong proteolytic action produced the greatest change in the electrophoresis pattern when compared to calf rennet (85). Cheesman (21) demonstrated the proteolytic nature of rennet and other proteolytic enzymes on casein on casein-agar gels plates. The proteolytic enzymes produced a clearing zone following one or more precipitation zones. Rennet diffused through the agar producing two precipitation zones and separated by a clear zone. Richardson (65) subsequently used this technique to differentiate among certain blends of enzyme coagulants. Ilany and Netzer (40) reported that it might be possible to reduce the proteolytic enzyme by means of heat, irridation, or special inhibitors, thereby making them more suitable for use as rennet substitutes. However, no results or specific methods were reported.

Cheesemaking properties. Results of cheesemaking trials are very scanty, and vary considerably for each bacteria enzyme. Veringa (86), Mann (50), and Babbar et al (9) reported that satisfactory cheese could be made from several of the microbial enzymes. However, manufacturing conditions must be thoroughly specified and carefully controlled. Cheeses

made in which there were deviations from the optimum conditions, resulted in bitter cheese (90). Srinivasan et al (75) reported that cheddar cheese made using the enzyme of B. cereus did not develop bitterness in 6 - 8 months of storage. Generally, the set time necessary to produce a firm curd was longer for the microbial enzymes than for calf rennet.

Camembert and cottage cheese had high moisture contents using the enzymes of Mucor pusillus var. Lindt (82). In other work using the Mucor rennet, Cheddar cheese was rancid and had a coarse mealy texture (10). Highly proteolytic microbial rennets resulted in elevated levels of NPN and a soft curd (84). There was an increase in whey fat when cheese was made using the enzyme of Mucor pusillus. At 14 months, the Cheddar cheese was slightly bitter having an NPN value of 42.0% of total nitrogen compared to 33.8% for rennet cheese (66).

In commercial scale cheesemaking trials, the Mucor enzyme when used alone and in combination with pepsin or calf rennet, produced satisfactory cheeses (59). The cheeses manufactured were Cheddar, Romano, Provolone, and blue. Irvine et al (42) reported excellent Cheddar cheese was made from the enzyme of B. subtilis. Some modifications were necessary, however, in the cheesemaking operation. Sweet cheeses were unsatisfactory because of the extensive proteolysis and off-flavor development. Curd yields also were lower due to the high proteolytic activity. Cheeses made using the enzyme of E. parasitica were judged to be equal or superior to control cheeses made with animal rennet (72). Characteristically, cheeses made using microbial enzymes age more rapidly than those using rennet.

Summary

Economic conditions as well as dietary and religious beliefs have dictated the need for a rennet substitute of nonanimal origin.

The potential higher plants and vegetable proteases which may be used are limited and relatively expensive to produce.

Microorganisms are the most readily available and most economic source of milk-clotting enzymes. They can be selected from a potentially infinite supply.

Microbial enzymes show enough variability, that by varying the processes of enzyme production and purification, they can be made suitable for use in cheese manufacturing.

EXPERIMENTAL PROCEDURES

Screening of Potential Microorganisms

Isolation and propagation. Fifteen cultures of unidentified bacteria, previously isolated from the air and known to coagulate milk, were obtained from Mr. V. D. Foltz, Kansas State University Division of Biology. A stock culture of each organism was propagated on proteose-peptone slants, consisting of 2% agar, 2% proteose-peptone, 0.5% sodium chloride, and 0.3% beef extract. The pH was adjusted to 7.0 and the medium autoclaved for 15 minutes at 121 C. At approximately six-week intervals, the stock cultures were transferred to new slants, incubated at 37 C for 24 hours, and subsequently stored at 4 C.

A routine check for purity of the stock cultures was performed periodically. Standard Plate Count Agar plates (79) containing 5% litmus milk were streaked from the stock cultures and grown at 37 C for 24 hours. Several representative colonies of each culture were picked into sterile litmus milk and incubated under the same conditions. Purity of the litmus milk cultures was ascertained by observation of the litmus milk, streaking on Standard Plate Count Agar, and microscopic examination. The actively growing organisms then were transferred to proteose-peptone slants, incubated as above and thereafter stored at 4 C.

Preparation of the cultures for milk-clotting enzyme production was carried out in a three-step transferring procedure, to assure that the cells were young and actively growing when inoculated into the enzyme production medium. A saline suspension of the stock cultures was made, the organisms

transferred to new slants, and incubated for 24 hours at 37 C. The cells on the new slant then were harvested in another saline suspension and transferred to 100 ml of proteose-peptone broth (4% proteose-peptone, 0.5% sodium chloride, 0.3% beef extract, in 100 ml of 0.2 M phosphate buffer at pH 6.6 in a 500 ml Erlenmeyer flask. The contents were grown on a water bath shaker (110 rpm's) at 30 C for 24 hours. The broth containing the cells was either centrifuged (6000 x g for 20 min) and the cellular paste used as the inoculum, or the broth was used directly in varying amounts.

Exploratory investigations. The fifteen bacterial strains were examined in preliminary studies for the production of milk-clotting enzymes, in an effort to select one or more strains for subsequent enzyme studies. Five different media were examined for their suitability in growing the microorganisms for the elaboration of milk-clotting enzymes.

Medium No. 1

Nonfat Dry Milk	100.0 g
Distilled Water	1 l
pH 6.3	

Medium No. 2

Dried Cottage Cheese Whey	100.0 g
Sodium Phosphate Buffer (0.2 M, pH 6.2)	1 l
pH 6.0	

Medium No. 3

Proteose-Peptone	40.0 g
Beef Extract	3.0 g
Sodium Chloride	5.0 g
Sodium Phosphate Buffer (0.2 M, pH 6.6)	1 l
pH 6.5	

Medium No. 4

Dried Cheddar Cheese Whey	100.0 g
Sodium Phosphate Buffer (0.2 M, pH 6.5) pH 6.4	1 l

Medium No. 5

Wheat Bran	60.0 g
Ammonium Sulfate	1.0 g
Magnesium Sulfate	0.2 g
Sodium Phosphate Buffer (0.2 M, pH 6.6) pH 6.5	1 l

These media were dispensed in Erlenmeyer flasks, sterilized by autoclaving at 121 C for 15 minutes, cooled, and inoculated with the cellular paste or liquid culture.

The inoculated media were incubated at the desired temperature, usually 30 - 35 C, for periods up to 10 days. Volume of media and size of flask were varied from 100 ml broth per 500 ml flask to 500 ml broth per 1000 ml flask. In addition, the cultures were grown quiescently in an incubator and by shaking on a water bath shaker at 110 rpm's. The media were assayed periodically, usually every 24 hours, for milk-clotting enzyme activity, total proteolytic enzyme activity, and extent of proteolysis of the substrate, by methods subsequently described. The pH of the media also was recorded at the same time intervals. In some of the trials, an estimate of the number of organisms present during the incubation period was determined by Standard Plate Count (78).

Selection of an Active Milk-Clotting Microorganism

After preliminary studies, the following procedures were adopted for selection of active microorganisms, and for studying the elaborated enzymes.

Each of the fifteen strains of microorganisms was inoculated into individual 100 ml volumes of sterilized wheat bran medium in 500 ml flasks. Incubation was carried out at 30 C on a mechanical shaker for 72 hours. At 24 hour intervals, samples of the media were analyzed for milk-clotting enzyme activity and total proteolytic enzyme activity. The pH change of the media was recorded. Additionally, individual lots of skimmilk were treated with the enzyme-containing broths and allowed to react for 24 hours. The treated lots of skimmilk were subsequently analyzed for extent of protein breakdown by vertical polyacrylamide-gel electrophoresis. Electrophoresis gel patterns of the enzyme treated skimmilk were compared to gel patterns of rennet treated skimmilk, unhydrolyzed skimmilk, and whole casein standards by visual and densitrometric analysis. The elaborated enzymes were further analyzed by placing a sample of each enzyme-containing broth on casein-agar gel plates, and comparing the zonal patterns of precipitation and proteolysis to a rennet standard.

From results of these analyses, four cultures whose milk-clotting enzymes most closely resembled the action of calf rennet were selected for further evaluation. Evaluation of the enzymes elaborated by the four organisms was based upon the same tests and techniques as outlined above. The enzyme-elaborating culture showing the greates potential as a rennet substitute was further selected from this evaluation.

Studies on the Selected Culture

The effect of several variables on the milk-clotting enzyme production was studied when the selected culture was grown in wheat bran medium. The studies were aimed at enhancing milk-clotting enzyme production by ascertaining the optimum conditions of the growth medium. In all the studies, the culture was grown under submerged conditions on a mechanical shaker.

Determination of effect of calcium phytate. The wheat bran medium was supplemented with phytic acid in the form of the calcium salt to the extent of 1% to 8% w/v. Five-hundred ml Erlenmeyer flasks containing 250 ml of sterilized media were inoculated with the test culture and incubated at 30 C for periods up to 192 hours. At intervals during incubation, milk-clotting and total proteolytic enzyme activities were checked. In some trials, Standard Plate Counts were used to determine the number of organisms present during the incubation period.

Determination of effect of calcium ions. The wheat bran medium was supplemented with 6% calcium ions from three different sources, including calcium chloride, calcium phosphate, and calcium phytate. The media were sterilized by autoclaving and inoculated with the test organism. Incubation was continued for a period of 120 hours and milk-clotting and total proteolytic enzyme activities were checked periodically.

Determination of effect of pH. Inoculated wheat bran medium were buffered to pH values of 5.2, 6.2, 6.6, and 7.2 with either sodium acetate or sodium phosphate buffers at 0.5 M. No adjustment of pH was made during the 72 hour period of incubation in which the media were checked for milk clotting and total proteolytic enzyme activities at regular intervals.

Determination of effect of temperature. Inoculated flasks of wheat bran medium were placed at temperatures of 25, 30, 37, and 45 C. Incubation was continued for a period of 72 hours and the media checked at regular intervals for enzyme activity.

Determination of effect of age of bacterial culture. An equal weight of cellular paste of 24 hour and a 96 hour old culture of the test organism was introduced into 250 ml quantities of wheat bran medium and incubated for 120 hours. The media were checked at 24 hour intervals for enzyme activity, and Standard Plate Counts were made to ascertain probable numbers of bacterial cells. Additionally, an equal weight of the cellular paste of the 24 and 96 hour old cultures was mixed with sterile powdered alumina and ground with mortar and pestal--to disrupt the cells--and introduced into the wheat bran medium. The media were tested at zero hours of incubation to ascertain enzyme activity.

Identification of the Selected Culture

The organism selected was observed to be a gram variable nonsporeforming rod. Further characteristics of the organism were determined by some of the criteria according to Bergey (15). The organism was tentatively placed in the genus Pseudomomas.

Isolation of the Enzyme

The selected culture was grown under optimum conditions, as determined above, to produce the milk-clotting enzyme in quantity and to further isolate, concentrate, and characterize the enzyme.

Production. One-hundred ml of liquid broth containing the selected culture were inoculated into 3 liters of wheat bran medium. The wheat bran was previously sterilized in a thin layer for 60 minutes. It then was mixed with the phosphate buffer and other ingredients and autoclaved for an additional 60 minutes in a fermenter jar. Incubation was carried out in a Brunswick Fermenter (Model F-5) for the desired period of time. The temperature was controlled at 32 C by menas of a heated water bath, into which the fermenter unit was placed. Aeration of the ferment was achieved by passing forced air through a glasswool filter and into the fermenter at the rate of 2 ℓ per minute. Maximum agitation of the growth medium was necessary for highest enzyme production. Agitation was controlled at 475 rpm's. Dow Antifoam FG-10 was added periodically to the ferment, when foaming became excessive.

To follow the progress of enzyme production, the regular tests for enzyme activity were employed. The pH of the ferment was maintained between 6.4 to 6.8 by addition of either 3 N sodium hydroxide or 2 N hydrochloric acid. The ferment also was streaked periodically to ascertain viability and purity of the culture organism.

The optimum length of incubation was ascertained in a preliminary trial by checking enzyme activity at 24 hour intervals.

Concentration. After the culture had incubated for the desired period of time, the ferment containing the enzyme was filtered through a double layer of cheese cloth to remove extraneous wheat bran. The filtrate was centrifuged at 6000 x g for 20 minutes in a refrigerated centrifuge to remove cellular and other solid material. The centrifuged extract was

concentrated by flash evaporation under vacuum at 30 C to one-half the original volume. The concentrated extract then was dialyzed against cold distilled water for 24-36 hours to remove the dissolved buffer salts. Dialysis water was completely changed every four hours. The salt-free enzyme-containing broth was further concentrated under vacuum to approximately 10% the original volume. The concentrated enzyme was lyophilized, and subsequently stored in the cold for future studies.

At appropriate intervals during the isolation and concentration procedures, samples were taken for determination of milk-clotting and total proteolytic activities. Additionally, semi-micro Kjeldahl nitrogen determinations were made on the same samples for calculation of specific activities. A record of the volume at the various stages of concentration was kept for determination of total units of activity, and fold-purification of the enzyme preparation.

Analytical Methods

Milk-clotting activity. The procedures used for assay of milk-clotting activity were a modification of the method used by Ernstrom (29) and by Srinivasan et al (77). Low heat nonfat dry milk (NDM) reconstituted to 12% in distilled water was used as the substrate. It was fortified with 0.02 M calcium chloride, and was allowed to equilibrate for 20 hours at 5 C before use. One crystal of thymol was added and the substrate stored at 5 C. New substrate was prepared weekly. For assay of enzyme activity, 10 ml of the substrate in a 125 ml wide mouth bottle was tempered at 40 C for 5 minutes. Two ml of the enzyme-containing broth of a suitable dilution were added. The time clotting to occur at 40 C was measured on an

apparatus described by Sommer and Matsen (73). The first appearance of visible flecks on the moving glass surface was taken as the end point. Under these conditions of assay, the time of blotting was found to be inversely proportional to the enzyme activity. Milk-clotting enzyme activity was calculated in units of activity per ml of broth as represented by 60/t x D.F. The symbol "t" represented time in seconds, and D.F. designated the dillution factor. For the lyophilized material, activity was expressed in wits per mg. A rennet standard of a 1:2000 dilution was run daily to check the substrate. In trials where applicable, milk-clotting activity was expressed in Rennet Units (R. U.) per ml as described by Ernstrom (29).

Total proteolytic activity. Proteolytic activity was determined by the procedures of Anson (3) as modified by Cole (22). One ml of the enzyme preparation was added to 5 ml of a 1% solution of casein. The samples were incubated at 37 C for exactly 30 minutes, before 5 ml of 11% trichloracetic acid (TCA) were added to stop the reaction and precipitate the unhydrolyzed proteins. The tubes were allowed to remain at 37 C for an additional 30 minutes. The contents then were filtered through Whatman No. 42 filter paper. The extent of proteolysis was measured by reading the absorbancy of the filtrates at 280 mμ in a Beckman Model DU spectrophotometer. Blanks were prepared by adding 5 ml of TCA to 5 ml of substrate followed by addition of 1 ml of the enzyme preparation. One unit of proteolytic enzyme activity was arbitrarily defined as the amount of enzyme that produces a 0.1 increase in absorbancy at 280 mμ in 30 minutes at 37 C.

The casein substrate was prepared by adding 10 g of vitamin-free casein to approximately 100 ml of 0.01 M sodium phosphate buffer, pH 6.6. Three ml of 3 N sodium hydroxide were added and the mixture heated in a

boiling water bath for 15 minutes to solubilize the casein. After cooling, the volume was adjusted to 250 ml with phosphate buffer, making a final concentration of 4% casein. Two-hundred mg of sodium azide were added as a preservative. The above preparation was diluted 1:4 in phosphate buffer for use.

Nitrogen determinations. For calculation of specific milk-clotting and total proteolytic activities, the nitrogen concentration of the enzyme preparations was measured by a semi-micro Kjeldahl precedure (38). A 2 ml sample of the liquid enzyme preparation or 200 mg of the lyophilized product was digested with 3 ml of concentrated H_2SO_4. One g of digestion catalyst--sodium sulfate and mercuric oxide--was added. The digested material was then distilled using 8 ml of 50% NaOH containing sodium thiosulfate. It was distilled into 25 ml of 2% boric acid solution containing Tashiro indicator. The boric acid solution was subsequently titrated with 0.03 N HCl. Nitrogen concentration was calculated in mg per ml of broth, or mg of nitrogen per mg of dried material.

Determination of specific activity. Since enzyme isolation involved the selective removal or concentration of other proteins, it was necessary to assess milk-clotting and total proteolytic activities relative to the amount of nitrogen present. Specific activities were obtained by dividing units of milk-clotting or total proteolytic activity per ml broth by mg nitrogen per ml broth. It was expressed in units of activity per mg of nitrogen.

Also calculated in the isolation and concentration procedures were total units of milk clotting and total proteolytic enzyme activity, enzyme

yield, and fold-purification. These calculations were made according to procedures of Dixon and Webb (23).

Casein-agar plate assay. This method for testing enzyme activity was a modification of the procedures of Cheesman (21). The casein-agar gel was prepared as described by Ganguli and Bhalerao (33). The substrate contained 1% Hammerstein casein, 1% agar, and 0.1% sodium acetate, and 10 mM calcium chloride. Solutions of casein and agar were prepared separately to avoid exposure of casein to boiling temperature necessary to melt the agar. Twenty ml of the pH 5.8 substrate were solidified in a petri dish. A 12 mm diameter well was cut into the agar and filled with 0.1 ml of the enzyme preparation. One drop of toluene was added to stop microbial growth. The upright petri dishes were incubated at 30 C and observed after 24 hours for zones of precipitation and proteolysis. Rennet standards of a suitable dilution were run concurrently for comparison.

Electrophoresis of enzyme-treated milk. To ascertain the proteolytic nature of the bacterial enzymes on raw skimmilk, vertical polyacrylamide-gel electrophoresis was performed. A 1 ml aliquot of the enzyme preparations was pipetted into 100 ml of fresh raw skimmilk. Toluene was added to stop any microbial growth. The skimmilk then was incubated at 37 C for 23 hours. At the end of the incubation period, a 1 ml sample, or equivalent amount of coagulum, was combined with 2 ml of 9 M urea, 1 drop of 2-mercaptoethanol, and 6 drops of dye marker. The sample mixture was allowed to equilibrate for 1 hour before applying to the electrophoresis gel.

Vertical polyacrylamide-gel electrophoresis of the enzyme-treated skimmilk was performed according to the procedures described by the E-C Apparatus Corporation (25). The gel was prepared using a 9% solution of

cyanogum-41 dissolved in a Tris-HCl buffer at pH 8.9. Two tenths ml of tetramethyethylenediamine (TMED) and 0.02 ml Photoflow wetting agent were added to 200 ml of the mixture. For use, 75 ml of the above preparation was combined with 75 ml of 9 M urea containing 0.1 g of ammonium persulfate (AP) catalyst. The mixture was poured between the cooling plates of the cell and allowed to polymerize. The electrode buffer used was Tris-glycene, pH 8.3, prepared by dissolving 1.2 g Tris and 5.8 g glycine in water and making up to 2 ℓ (35).

Five-hundredths ml of the sample mixtures was pipetted into the slots of the gel. Electrophoresis was run at 200 volts for 4-6 hours or until the dye marker had migrated a total of 10 cm. Skimmilk, casein, and rennet treated skimmilk were run as standards for comparison.

The gels were stained with 0.2% Amido Black 10-B in equal parts of methanol and 20% acetic acid for approximately 10 minutes. They then were destained with 7% acetic acid over a period of several days.

Densitometer analysis. The stained electrophoresis gels were analyzed densitometrically for extent of protein breakdown, with particular attention being paid to the β-casein band. Densitometer scannings were made on a Photovolt Densitometer which was set at maximum sensitivity. The baseline was set at 10% for the gels.

The proteins bands were evaluated from densitometer scans using the classification of Morr (57) and Thompson et al (80). A semiquantitative scale ranging from minus 5 to plus 5 was used for evaluation. Minus values indicated progressive diminution of the peak area, due to protein breakdown; while plus values indicated progressive increases in the peak area resulting from protein hydration or combining of protein fragments.

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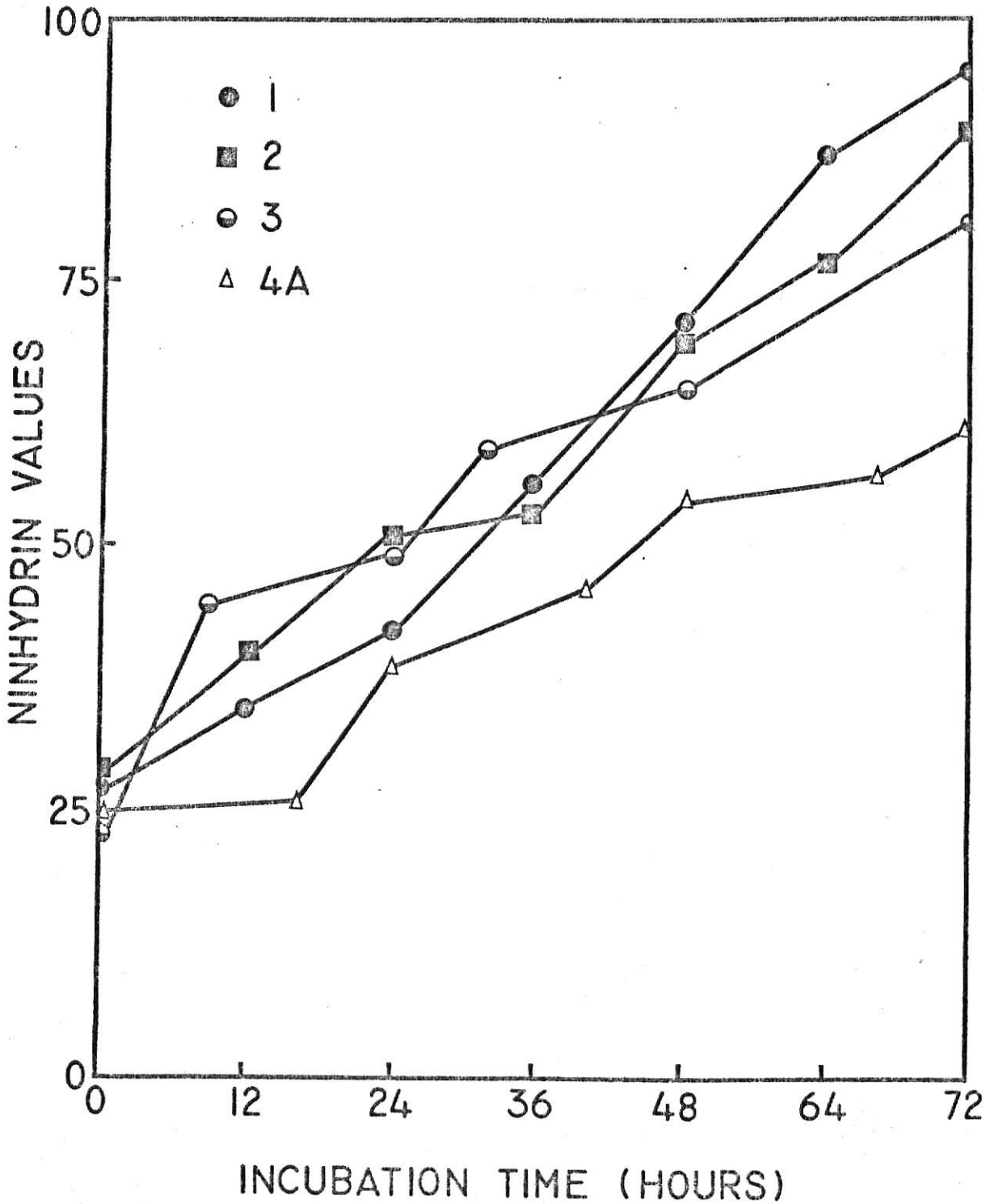

Figure 1. Relative amounts of proteolysis produced by isolates 1, 2, 3, and 4A, as examples of the 15 cultures, in 10% solids reconstituted non-fat dry milk. The cultures were grown under submerged conditions at 30 C.

RESULTS AND DISCUSSION

Screening of Potential Microorganisms

Temperature studies. Fifteen pure bacterial cultures, isolated from the air, were screened for their milk-clotting enzyme properties. To determine the optimum temperature for growth, the isolates were grown initially in litmus milk cultures at 25, 32, 37, and 45 C. Generally, all isolates showed a wide range of growth temperatures. The temperatures at which the litmus milk showed the greatest amount of change are denoted by a double plus (++) in Table 1. The litmus milk reactions for most isolates were alkaline proteolysis, reduction, and coagulation. Since all isolates grew at 30 C, this temperature was used for incubation for the duration of preliminary screening.

Selection of a suitable growth medium. In other preliminary studies, the isolates were cultivated in 100 ml of reconsrituted nonfat dry milk in 500 ml Erlnmeyer flasks. The cultures were incubated for 72 hours at 30 C in surface (quiescent) as well as submerged (shaken) conditions. Milk-clotting activities are reported in Table 2. The enzyme activity is expressed as units of activity per ml of broth according to the formula under "Experimental Procedures".

It can be seen in Table 2 that milk-clotting enzyme production under both submerged and surface conditions was very poor. Among the 15 cultures, maximum milk-clotting activity at the end of 72 hours of incubation was 0.2 units per ml of broth for isolate 11. When activities of surface and submerged conditions are compared, it can be seen that in

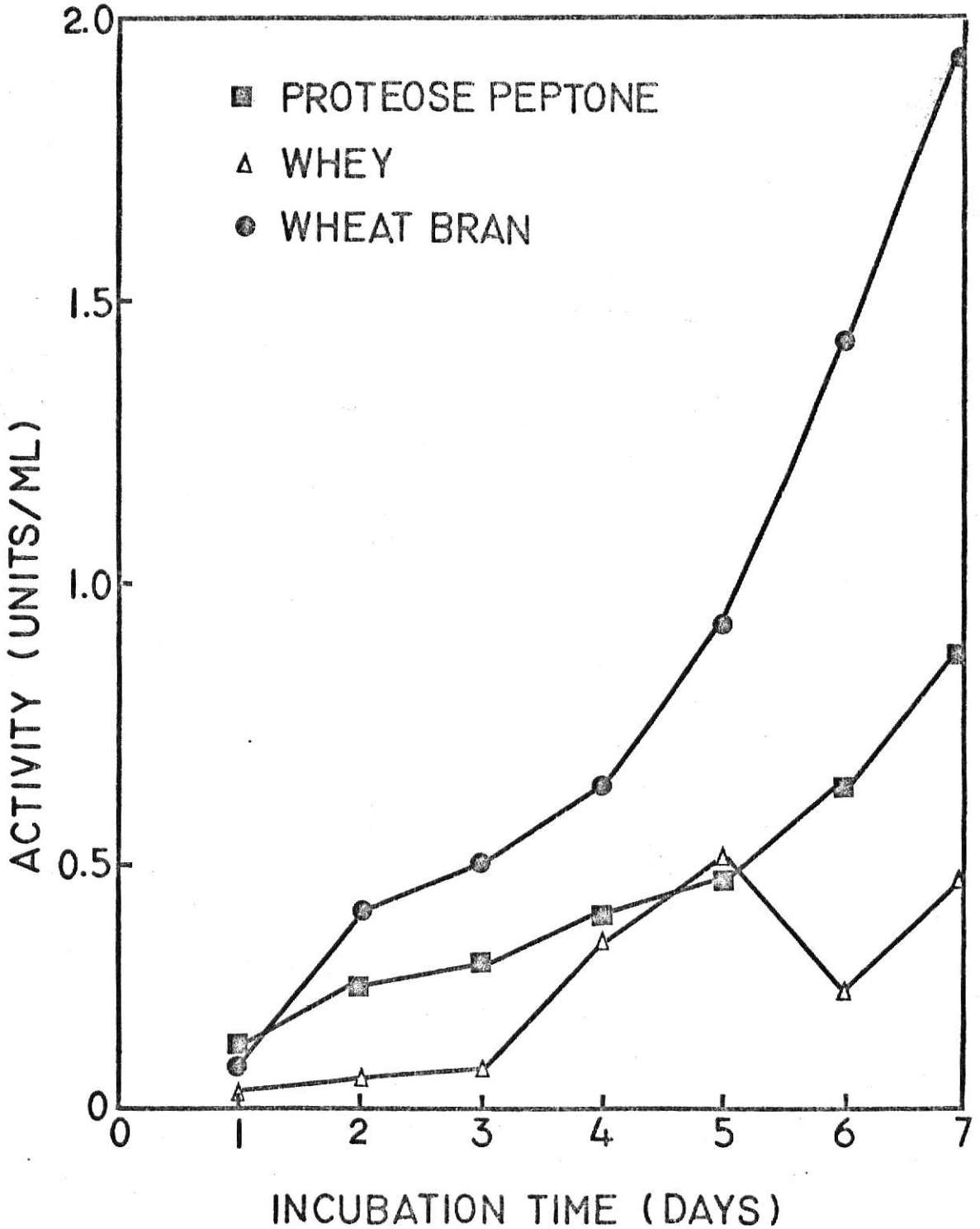

Figure 2. Total proteolytic activity of isolate 3, as an example of the 15 cultures, grown in proteose-peptone (medium no. 3), Cheddar cheese whey (medium no. 4), and wheat bran (medium no. 5). Incubation was at 30 C under submerged conditions.

general, submerged conditions were more conductive to enzyme production. Isoltes 4A and 11, particularly, showed a two-fold increase in enzyme activity when cultured by shaking. Exceptions were isolates 6 and 22B where there was an actual decrease in activity when the organisms were grown in submerged conditions.

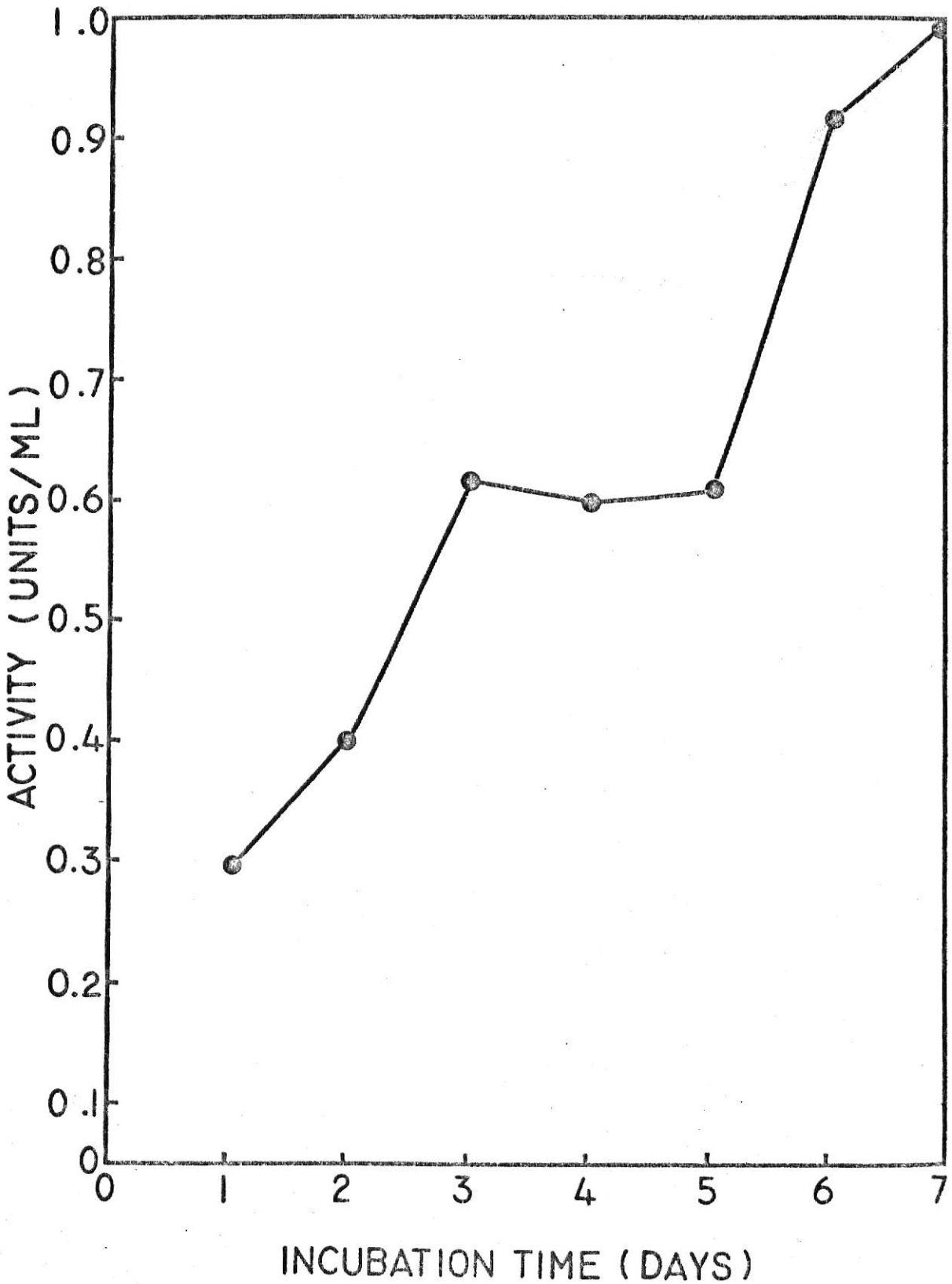

Figure 3. Milk-clotting activity of isolate 3, as an example of the 15 cultures, grown in wheat bran (medium no. 5) under submerged conditions at 30 C.

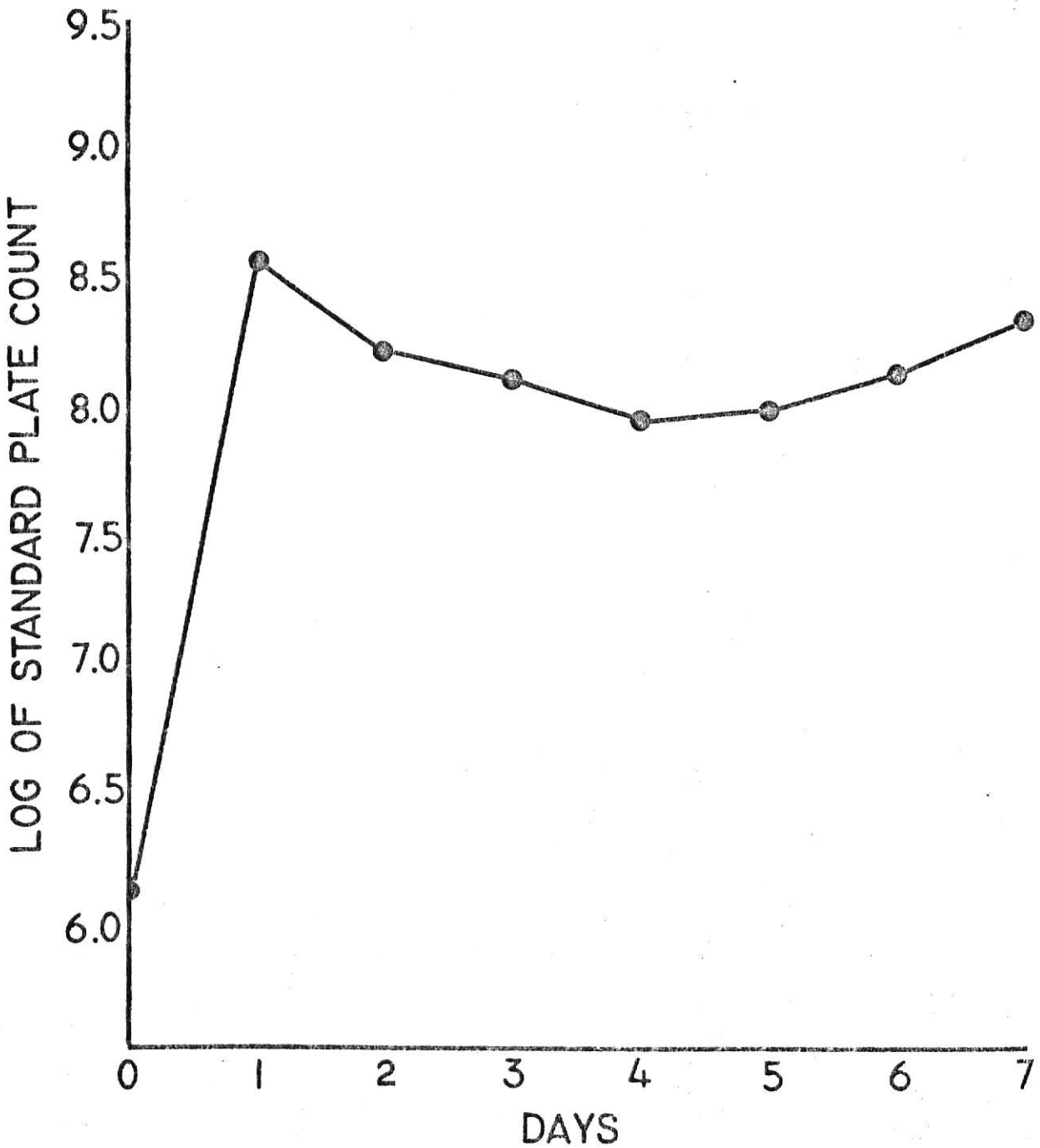

Figure 4. Growth curve of isolate 3, as an example of the 15 cultures, in wheat bran (medium no. 5) at 30 C under submerged conditions.

Table 1. Temperature studies of bacterial isolates grown in litmus milk cultures.

	Growth at degrees C			
Isolate no.	25	32	37	45
1	0[a]	+[b]	++[c]	+
2	±	++	++	±[d]
3	+	++	+	+
4A	±	+	±	±
4B	±	+	±	±
5	±	+	+	0
6	0	+	±	0
9A	++	++	+	0
9B	++	+	±	±
11	0	+	+	±
16	0	+	+	++
17	±	+	++	+
19	±	+	++	+
22A	++	+	+	0
22B	+	++	+	+

[a] No growth

[b] Growth

[c] Apparent optimum

[d] Doubtful growth

Table 2. Milk-clotting activity of bacterial isolates grown in surface and submerged conditions in medium no. 1.

Isolate no.[b]	Milk-clotting activity[a]	
	Submerged (shaken)	Surface (quiescent)
1	.130	.122
2	.189	.181
3	.101	.080
4A	.143	.071
4B	.111	.094
5	.138	.105
6	.119	.140
9A	.110	.104
9B	.106	NC[c]
11	.208	.119
16	.104	.101
17	.104	.092
19	.108	.096
22A	.119	.090
22B	.094	.109

[a]Expressed in units of activity per ml of broth.

[b]Isolate numbers as per received from the Department of Biology.

[c]Denotes no clotting.

Suitability of the skimmilk medium for milk-clotting enzyme production was further assessed by means of the quantitative ninhydrin test. In most cases, quantitative ninhydrin provides an indirect measure of proteolytic activity by determining the amount of protein breakdown. Most milk-clotting enzymes are somewhat proteolytic in nature. Therefore, ninhydrin measured the amount of proteolysis resulting from bacterial metabolism and enzyme

EXPLANATION OF PLATE I

Casein-agar gel plates of commercial rennet extract and bacterial enzymes of the low, medium, and high milk-clotting activity groups.

Fig. 1. Rennet extract, undiluted (0) and 1:100 dilution.

Fig. 2. The enzyme preparation of 72 hours of incubation of isolate 1 (medium group) and 1:2000 dilution of rennet extract (R).

Fig. 3. The enzyme preparation of 72 hours of incubation of isolate 6 (low group) and 1:2000 dilution of rennet extract (R).

Fig. 4. The enzyme preparation of 72 hours of incubation of isolate 16 (high group) and 1:2000 dilution of rennet extract (R).

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PLATE I

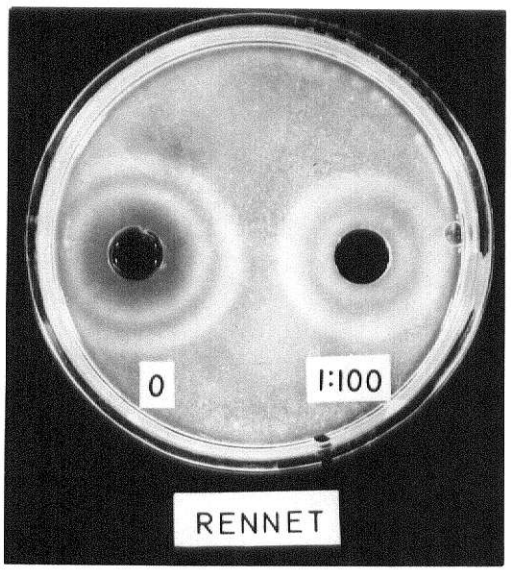


Figure 1

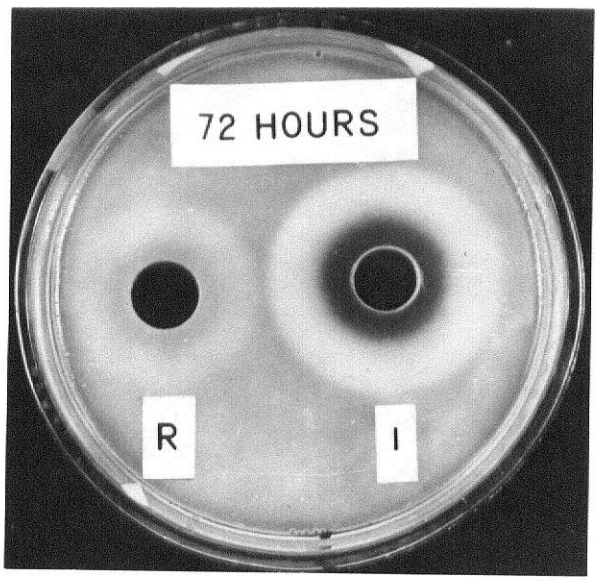


Figure 2

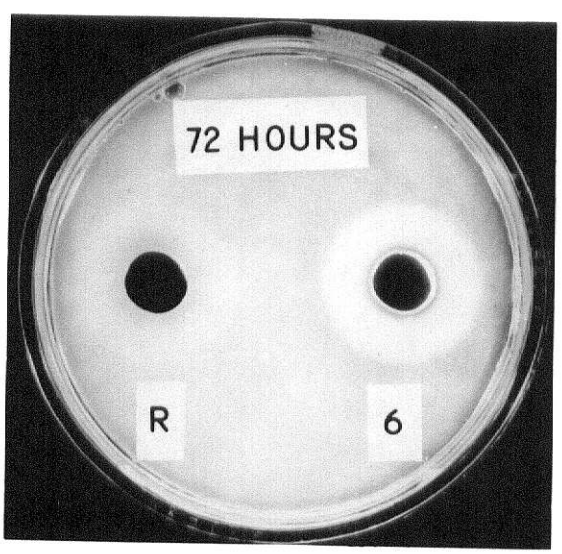


Figure 3

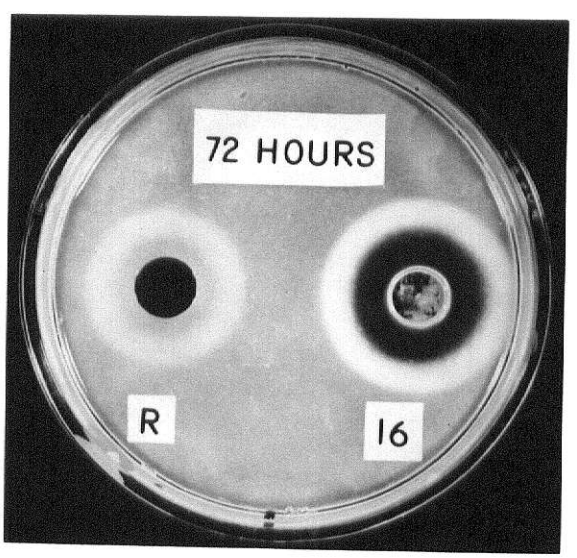


Figure 4

elaboration. Figure 1 illustrates ninhydrin values for the 72 hour incubation period in which selected examples of the 15 organisms were grown in skimmilk (medium no. 1) under submerged conditions at 30 C. For the four cultures studied, all values were relatively low when compared to ninhydrin values in fermentations with other organisms (79). Trials in which incubation was carried out for a total of seven days showed only slightly higher values than those reported in Figure 1.

It was concluded that milk medium was unsuitable for the production of milk-clotting enzymes. In later work, enzyme extraction by ammonium sulfate precipitation was unsuccessful because of the excessive amount of milk protein in the precipitate with the enzyme. Milk medium, however, was useful in preliminary screening for determining which bacterial strains elaborated milk-clotting enzymes.

In other preliminary trials involving various alternative media aimed at increasing milk-clotting enzyme activity, two kinds of whey media, a proteose-peptone medium, and a wheat bran medium were studied. Trials were made with variation in pH, temperature, incubation time, size of inoculum, volume of substrate, and size of flask. The cultures were grown under both submerged and surface conditions. Tests were made on milk-clotting and total proteolytic activity.

With the exception of the wheat bran medium, the other media tested were unsatisfactory for milk-clotting enzyme production. It was found that most isolates grew best under submerged conditions of maximum aeration and agitation. Figure 2 depicts the total proteolytic activity of a selected example of the 15 isolates grown in proteose-peptone (medium no. 3), Cheddar

cheese whey (medium no. 4), and wheat bran (medium no. 5). While all activities remained low over the seven-day period, highest proteolytic activity was exhibited in the wheat bran medium. Both buffered and unbuffered whey medium became progressively acid during the incubation period. It was speculated that the low pH was inhibitory to enzyme production, or destroyed the elaborated enzyme.

Casein-agar gel plates, employed as a crude screening test for enzyme activity, were unsatisfactory for the whey medium. The low pH of the medium caused acid precipitation of the suspended casein.

Figure 3 depicts the milk-clotting activity for isolate 3, as an example of the 15 cultures, grown in wheat bran medium under the same conditions as above. With the exception of the three to five-day incubation period, milk-clotting activity steadily increased for the entire period. Activity, however, was not as high as in later trials because of low aeration and agitation. Milk-clotting activity in all other media was substantially lower than for the wheat bran medium. Indeed, the milk-clotting activity in the wheat bran medium reporesented a 100-fold increase over other media.

Standard Plate Counts were used to follow cell poliferation in the various media during incubation. Counts were very similar for all media. The highest visible cell count in wheat bran for isolate 3, as an example of the 15 cultures, grown under submerged conditions at 30 C was after one day of incubation. Figure 4 shows that the number of visible cells increased rapidly and maintained a rather constant plateau after the first day until the end of the test. This was the general pattern of other bacterial isolates grown under the same conditions.

Selection of an Active Milk-Clotting Microorganism

After the preliminary trials to determine optimum growth conditions, the 15 strains of bacterial isolates were qualitatively and quantitatively evaluated for their ability to produce milk-clotting enzymes. Studies were directed toward selecting one culture whose milk-clotting enzyme showed the most potential as a rennet substitute. Therefore, not only the organism's ability to produce the enzyme, but also the characteristic of the elaborated enzymes were of paramount importance.

The isolates were grown initially in proteose-peptone medium at 30 C under submerged conditions. The cellular material was gathered by centrifuging at 6000 x g for 20 minutes. Equal weights of the cell pastes then were inoculated into individual lots of wheat bran medium buffered at pH 6.6. Incubation was continued for 72 hours at 30 C on a mechanical shaker. Conditions of substrare pH and incubation temperature were so chosen to parallel conditions of the milk in a normal cheesemaking operation.

Milk-clotting activity. At 24 hour intervals, the enzyme preparations were assayed for milk-clotting activity. Activities expressed in units per ml of broth are shown in Table 3.

Table 3. Milk-clotting activity of bacterial isolates grown in wheat bran (medium no. 5) for 72 hours at 30 C. Isolates categorized into low, medium, and high activity groups.

	Isolate no.	Milk-clotting activity[a] Incubation period at 30 C 24 hours	48 hours	72 hours
Low	2	.103	.033	NC
	4A	NC[b]	NC	NC
	5	NC	NC	NC
	6	NC	NC	NC
	17	NC	NC	NC
	19	NC	NC	NC
Med.	1	NC	.295	2.720
	3	.431	1.395	1.360
	9A	.229	.555	.540
	9B	1.444	2.950	2.850
	11	NC	1.575	1.390
High	4B	3.000	3.425	4.750
	16	5.800	10.200	8.700
	22A	8.275	6.950	6.450
	22B	5.000	4.500	5.000

[a]Expressed in units per ml of broth.

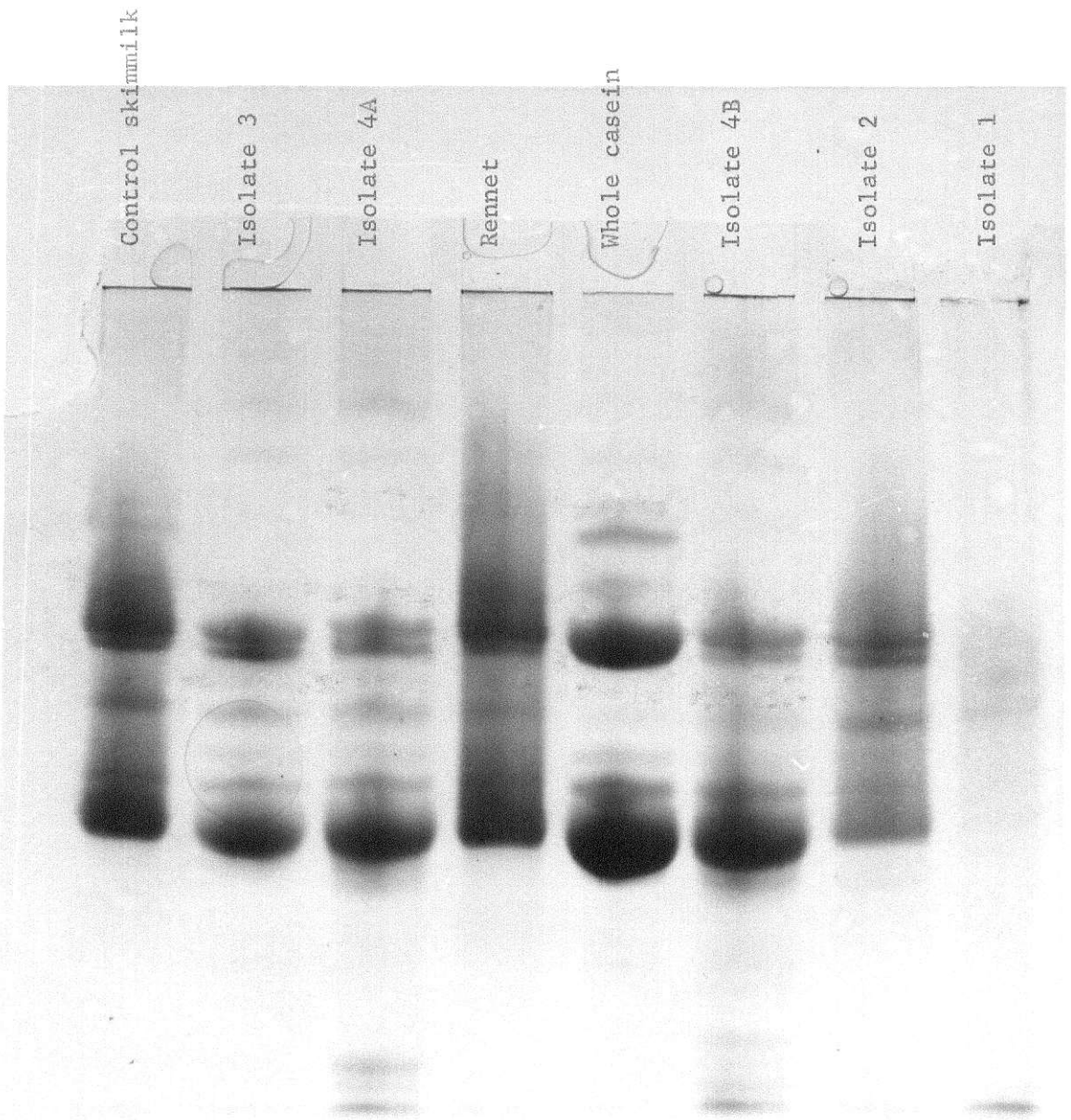
[b]No clotting occurred in 30 minutes.

On the basis of these results, the isolates were categorized into low, medium, and high activity groups.

EXPLANATION OF PLATE II

Vertical polyacrylamide-gel electrophoresis patterns of skimmilk treated with the enzyme preparations of 72 hours incubation of bacterial isolates 1, 2, 3, 4A, and 4B, control skimmilk, rennet treated skimmilk, and whole casein.

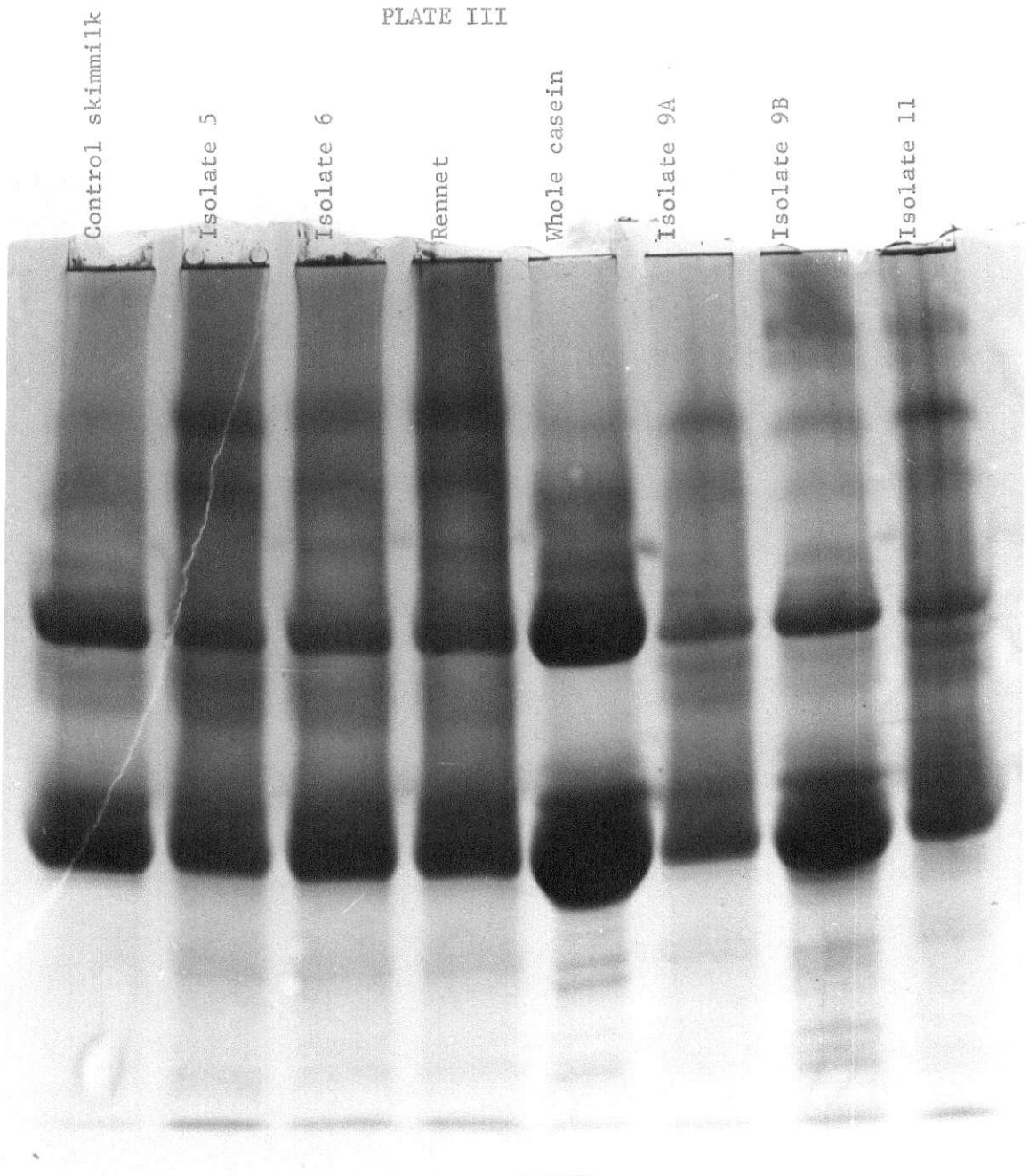
PLATE II


Control skimmilk
Isolate 3
Isolate 4A
Rennet
Whole casein
Isolate 4B
Isolate 2
Isolate 1

EXPLANATION OF PLATE III

Vertical polyacrylamide-gel electrophoresis patterns of skimmilk treated the enzyme preparations of 72 hours incubation of bacterial isolates 5, 6, 9A, 9B, and 11, control skimmilk, rennet treated skimmilk, and whole casein.

PLATE III


Control skimmilk
Isolate 5
Isolate 6
Rennet
Whole casein
Isolate 9A
Isolate 9B
Isolate 11

With few exceptions, isolates in the low group exhibited no milk-clotting activity during the 72 hours studied. Isolates of this group were considered unsatisfactory as sources of a rennet-like enzyme. Activities of the medium group ranged from 0.229 to 2.850 units per ml of broth, with an average of 1.772 units per ml of broth at 72 hours. These activities were essentially twice those shown by the low group. Activities of the high group were about three times the activities of the medium group, averaging 6.225 units per ml of broth at 72 hours. The high group invariably yielded loose textured curd in the milk-clotting substrate, while the medium group produced a firmer clot.

In most cases, milk-clotting activity increased with the length of time of incubation of the culture. Notable exceptions are isolates 2 and 22A in which activity actually decreased during incubation of the cultures. Other cultures exhibited maximum activity at 48 hours of incubation and then declined.

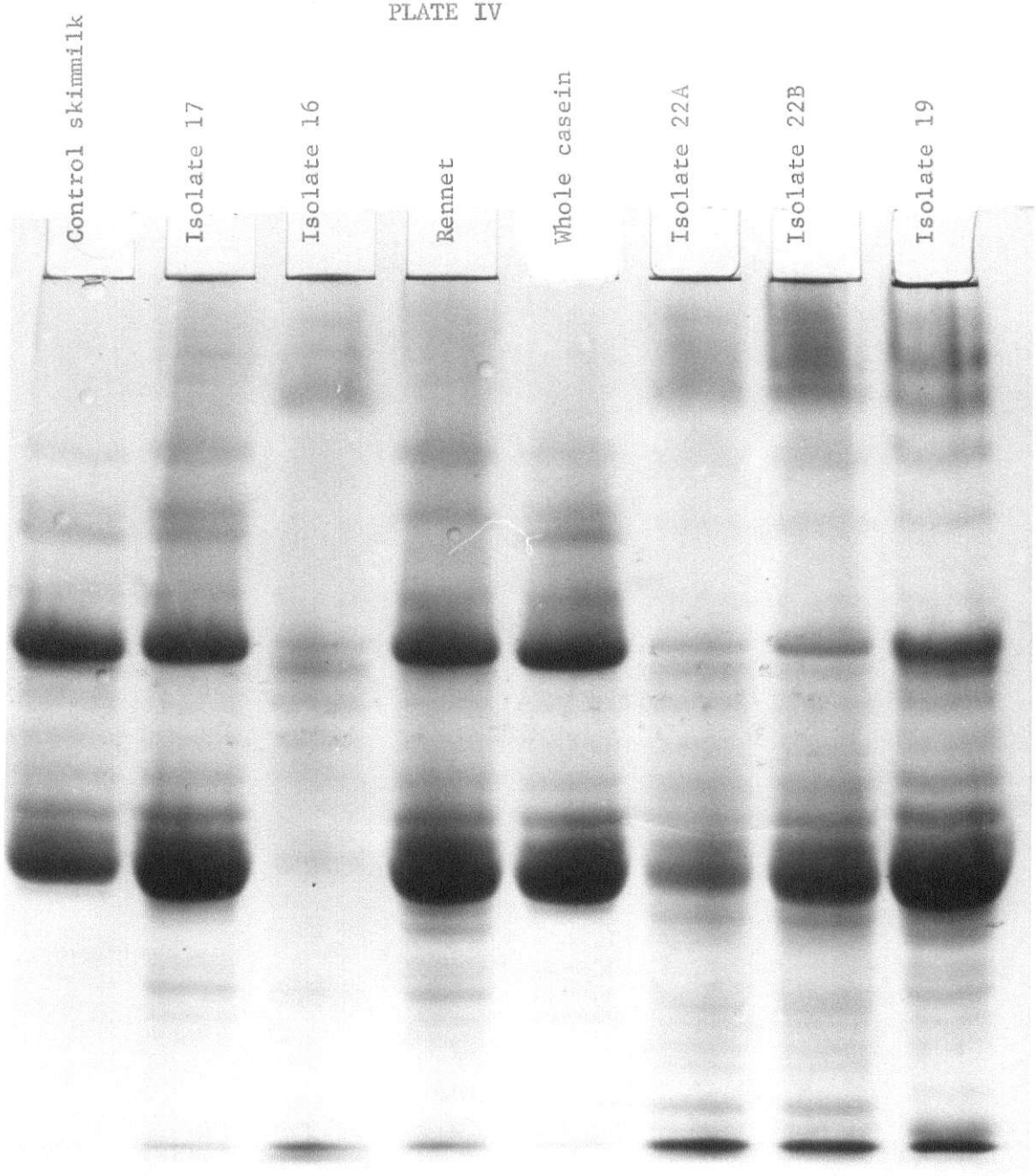
Milk-clotting activity also was calculated in Rennin Units per ml (R.U./ml) according to the procedures of Ernstrom (29). The standard rennet was arbitarily assigned a value of 100 R.U./ml at 40 C and diluted 1:2000 for testing. Although not presented in the table, in nearly all cases, milk-clotting activity of the bacterial enzymes was less than 2% of the activity of commercial rennet extract.

Casein-agar gels. In another screening test for rennet-like enzymes, the action of the bacterial enzymes was studied on casein-agar gel plates. An example of the low, medium, and high activity groups is shown in Plate I. As the enzyme preparations diffused through the agar, characteristic zones

EXPLANATION OF PLATE IV

Vertical polyacrylamide-gel electrophoresis patterns of skimmilk treated with the enzyme preparations of 72 hours incubation of bacterial isolates 16, 17, 19, 22A, and 22B, control skimmilk, rennet treated skimmilk, and whole casein.

PLATE IV


Control skimmilk
Isolate 17
Isolate 16
Rennet
Whole casein
Isolate 22A
Isolate 22B
Isolate 19

of precipitation and proteolysis were produced. Full strength rennet extract, seen in figure 1, produced two precipitation zones separated by a clear zone of proteolysis. Whereas, full strength rennet extract also showed an inner-most clear zone; in the 1:100 dilution rennet, this zone was absent.

When the enzyme preparations from the 72 hour fermentation were compared to a 1:2000 dilution of rennet extract, the bacterial enzymes differed only in the size of the zones. Generally, they produced one heavy precipitation zone and one proteolysis zone.

Isolate 6, seen in Figure 3, reporesenting the low milk-clotting activity group, showed one precipitation zone and only a small proteolysis zone. Isolate 1, in Figure 2, appeared more active than the 1:2000 dilution of rennet extract, showing larger zones of precipitation and proteolysis. It represented the medium milk-clotting activity group. Isolate 16, shown in Figure 4, representing the high milk-clotting activity group, primarily exhibited a large zone of proteolysis.

Total proteolytic activity. Of equal significance in these studies was the determination of proteolytic activity of the bacterial enzymes. A successful rennet substitute must possess a good ability to coagulate milk, without extensive proteolysis to the formed curd. Total proteolytic activity was measured in units of activity per ml of broth. These data are presented in Table 4. It can be seen that activities ranged from no activity to as high as 17 units per ml of broth. Generally, however, proteolytic activity of the bacterial enzymes was high when compared to rennet. A 1:2000 dilution of commercial rennet extract exhibited a proteolytic activity of 0.088 unit per ml at 37 C for 30 minutes.

The values representing proteolytic activity alone, mean very little in terms of the suitability of the enzymes as milk-clotting agents. However, when proteolytic activity is compared to milk-clotting activity some conclusions can be drawn. Generally, isolates having low proteolytic activity also exhibited low milk-clotting activity. Examples are isolates 6, 17, and 19. This indicated that milk-clotting and proteolytic enzymes were not produced by these cultures, or were not active under the growth conditions of this study. Furthermore, isolates showing high proteolytic activity and no milk-clotting activity were considered unsuitable as rennet substitutes. These correspond to other isolates of the low milk-clotting activity group.

Table 4. Total proteolytic activity of bacterial isolates grown in wheat bran (medium no. 5) for 72 hours at 30 C.

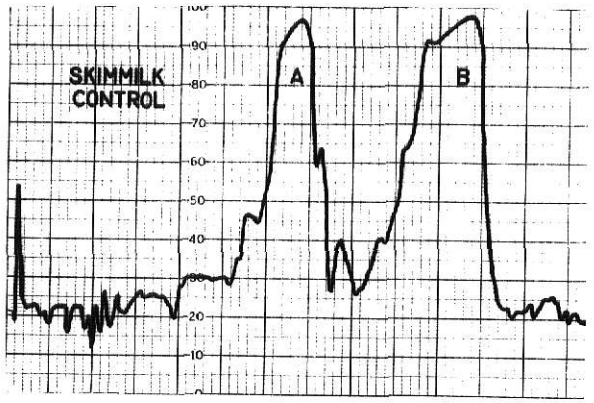
	Total proteolytic activity[a]		
	Incubation period at 30 C		
Isolate no.	24 hours	48 hours	72 hours
1	0.94	10.36	14.38
2	2.80	17.45	16.99
3	4.91	9.03	4.38
4A	1.05	1.11	10.89
4B	12.82	13.01	15.49
5	0.61	0.48	0.53
6	0.05	0.0	0.0
9A	2.48	5.27	4.72
9B	9.03	13.28	13.06
11	0.11	5.89	2.84
16	14.44	17.27	10.22
17	0.0	0.0	0.0
19	0.0	0.0	0.0
22A	16.83	16.88	15.27
22B	14.50	16.43	16.20

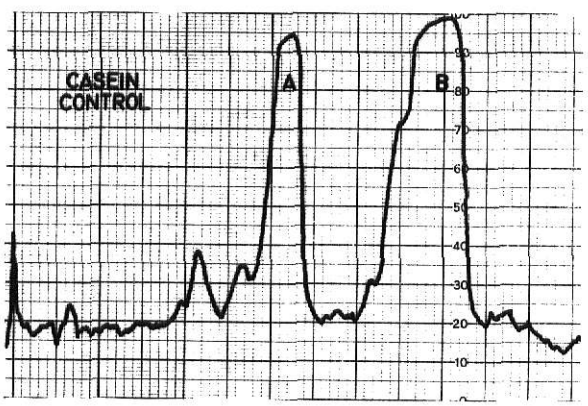
[a]Expressed in units of activity per ml of broth.

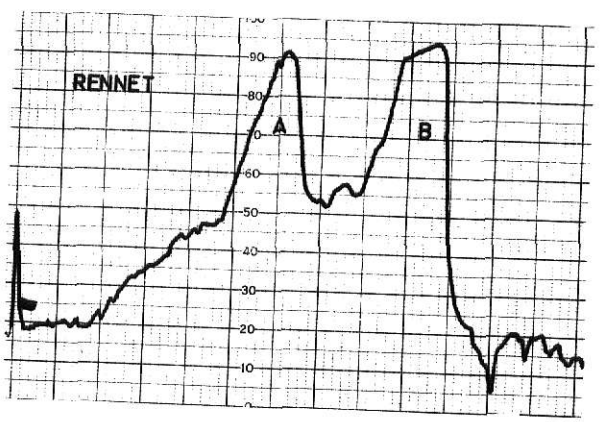
EXPLANATION OF PLATE V

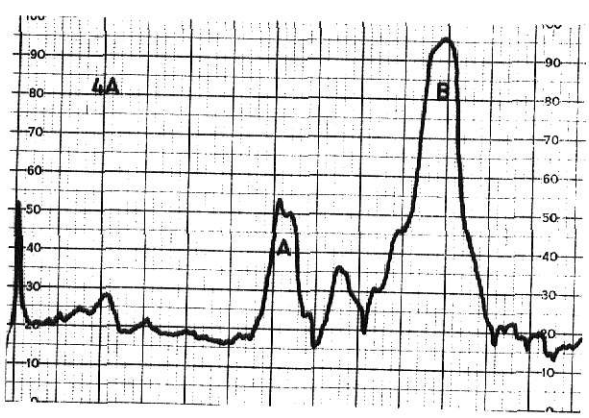
Densitometer scans of the electrophoretic patterns of skimmilk control, whole casein, rennet treated skimmilk, and skimmilk treated with the bacterial enzymes of isolates 4A and 19. The β-casein peak is designated "A", and α_s-casein is designated "B".

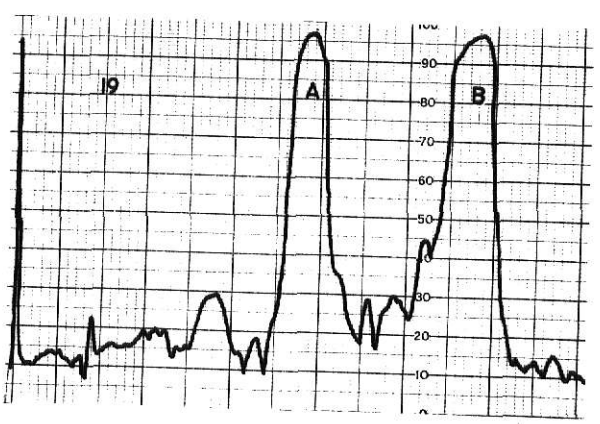
PLATE V











A number of the isolates exhibited very high proteolytic activity values. Isolates of the high milk-clotting activity group, while showing a good ability to coagulate milk, were considered unsatisfactory for most cheesemaking operations because of the extensive proteolysis that occurred after clotting.

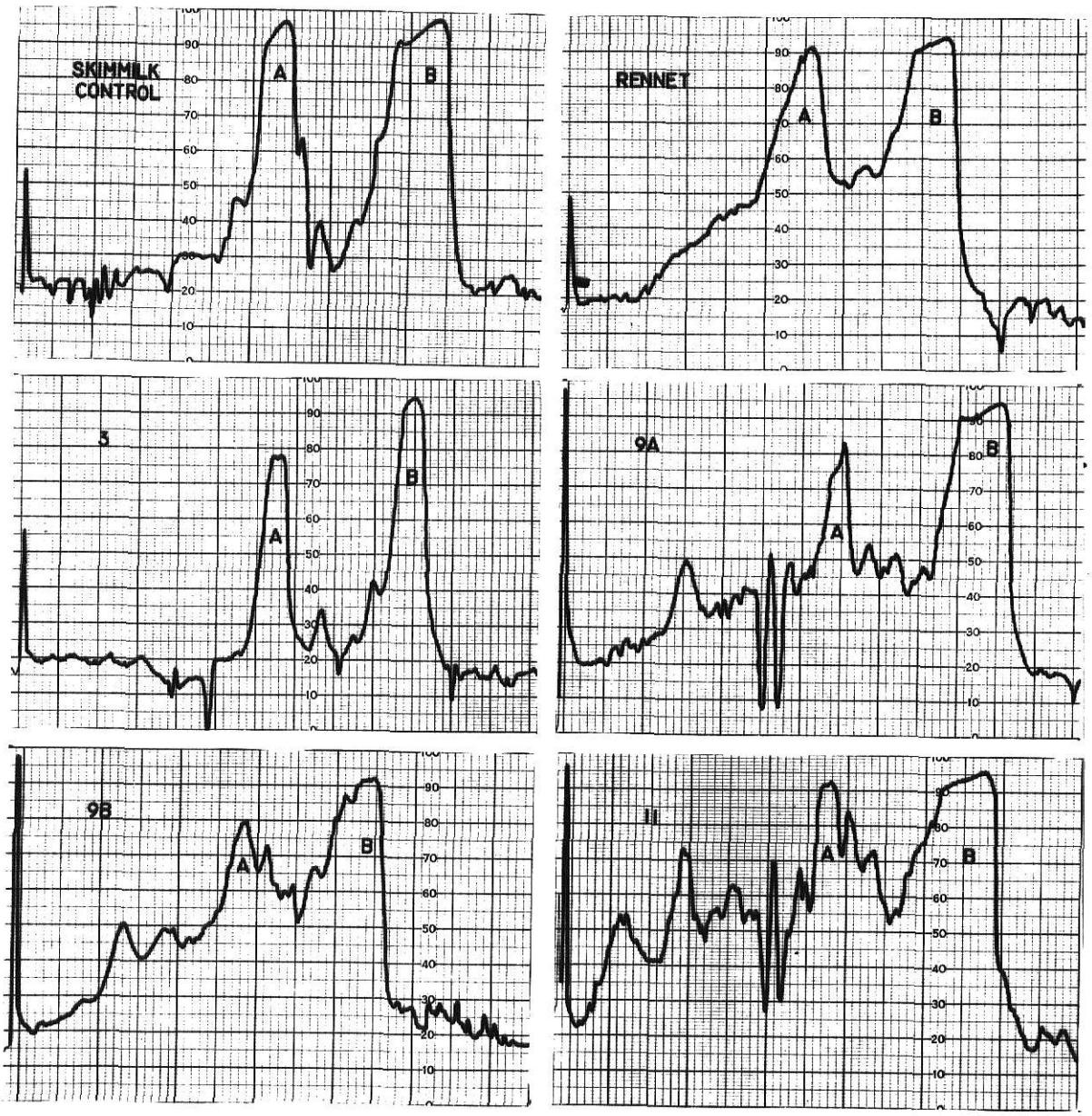
Based on a combination of the respective milk-clotting and total proteolytic enzyme activities, isolates 3, 9A, 9B, and 11 showed the most potential as possible rennet substitutes.

Effect of pH. Another significant observation in this study was the relationship of milk-clotting and total proteolytic activity to the change in pH of the growth medium. While the medium was buffered to pH 6.6, the pH still varied from a low of 6.1 to a high of 7.1 for certain cultures. In most instances the pH during the first day of incubation decreased, and on subsequent days the pH increased. Generally, the higher the pH value, the lower the milk-clotting activity, and the higher the total proteolytic activity. An example of this observation was isolate 2. The pH at 24 hours was 6.2 with milk-clotting and total proteolytic activity of .103 and 2.80 units per ml of broth respectively. At 72 hours, the pH was 6.7 and the enzyme exhibited no milk-clotting activity. The proteolytic activity was 16.94 units per ml of broth. The exact cause of the erratic changes in pH of the growth medium was not determined. It was speculated that fermentable sugars present in the wheat bran resulted in lactic acid production during the first stages of incubation. Buffering the medium at a higher concentration of salt to maintain the pH was detrimental to enzyme production.

EXPLANATION OF PLATE VI

Densitometer scans of the electrophoretic patterns of skimmilk control, rennet treated skimmilk, and skimmilk treated with the bacterial enzyme preparations of isolates 3, 9A, 9B, and 11. The β-casein peak is designated as "A" and α_s-casein is designated as "B".

PLATE VI



EXPLANATION OF PLATE VII

Casein-agar gel plates of the enzymes of isolates 3, 9A, 9B, and 11.

Fig. 1. The enzyme preparation of 72 hours incubation of isolate 3 and 1:2000 dilution of rennet extract (R).

Fig. 2. The enzyme preparation of 72 hours incubation of isolate 9A and 1:2000 dilution of rennet extract (R).

Fig. 3. The enzyme preparation of 72 hours incubation of isolate 9B and 1:2000 dilution of rennet extract (R).

Fig. 4. The enzyme preparation of 72 hours incubation of isolate 11 and 1:2000 dilution of rennet extract (R).

PLATE VII

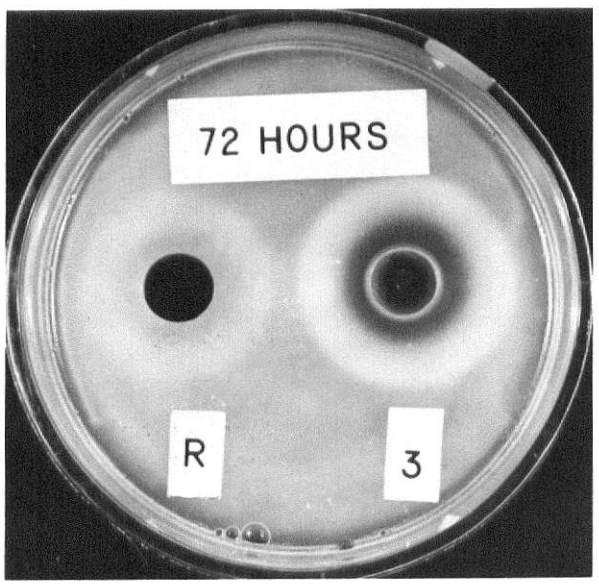


Figure 1

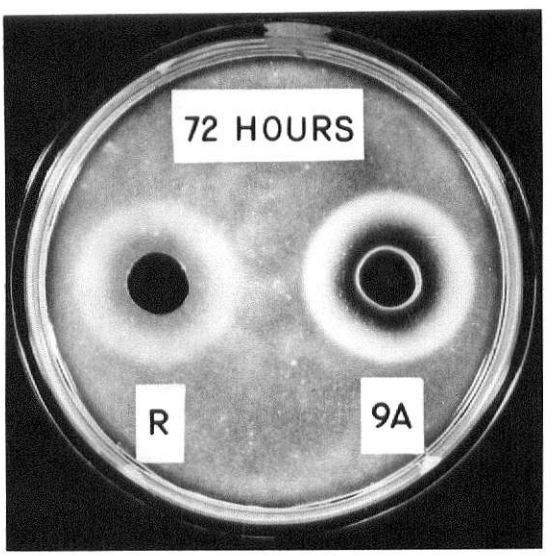


Figure 2

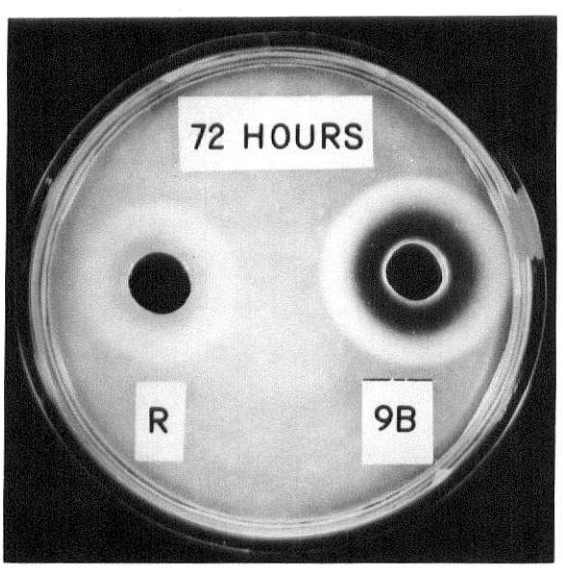


Figure 3

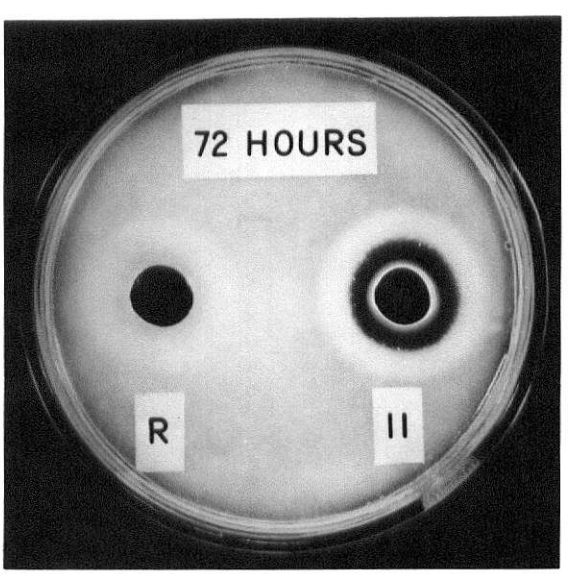


Figure 4

Electrophoresis of enzyme-treated skimmilk. The enzyme preparations were further studied using vertical polyacrylamide-gel electrophoresis. Particular attention was given to the extent of breakdown of the β-casein component, since it has been established by El-Negoumy that rennet action is nonspecific for β-casein (26). The data also provided information on the breakdown of other casein fractions due to enzymic hydrolysis. It should be noted that enzyme concentrations of the bacterial isolates were not at a standardized strength. The rennet was diluted 1:100, while the enzyme-containing broths were used undiluted. These unstandardized enzyme preparations no doubt presented some distortion when comparing one to another and to the rennet control. However, the electrophoresis analysis provided a qualitative evaluation of the bacterial enzymes in the screening process.

The electrophoretic patterns of raw skimmilk treated with the enzyme preparations of isolates 1, 2, 3, 4A, and 4B along with controls are shown in Plate II. Protein bands in the electrophoretic pattern of skimmilk were identified according to the work of Morr (57) and Thompson et al (80). The pattern of skimmilk treated with commercial rennet revealed primarily degradation products of α_s-casein moving faster than α_s-casein. The β-casein component exhibited no degradation, while κ-casein showed several slower moving components. Skimmilk treated with bacterial enzymes showed varying degrees of proteolysis toward all the protein fractions. In general, proteolysis by bacterial enzymes was extensive and nonspecific in nature.

With isolate 1, the electrophoretic pattern of skimmilk showed extensive proteolysis and no distinguishable protein bands. Nonspecific proteolysis was evident in the skimmilk pattern of isolate 2, in which the intensity of the α_s and β-casein bands were substantially reduced. The κ-casein band was missing in the skimmilk pattern of isolate 3, while the intensity of the β-casein band was only slightly diminished. The α_s-casein band in the pattern of isolate 3 was somewhat comparable to the control skimmilk and rennet-treated skimmilk patterns. The electrophoretic patterns of isolates 4A and 4B were very similar. They were characterized by substantial reduction of the β-casein and by the appearance of several fast moving components near the terminus.

Electrophoresis patterns of skimmilk treated with isolates 5 and 6 were very similar to the control milk as shown in Plate III. This was in agreement with data on milk-clotting and total proteolytic activity in which the isolates exhibited little to no enzyme activity. Both α_s and β-casein in the skimmilk pattern of 9A showed extensive proteolysis. The patterns of isolates 9A and 9B exhibited several fast moving components. Isolate 9B also showed a diffuse band near the origin and substantial diminution of the β-casein component. The enzyme preparation of isolate 11 was similar to rennet in its action on skimmilk. A slow moving component was observed near the origin, while α_s and β-casein showed only slight proteolysis.

When the enzymic action of isolates 16, 17, 19, 22A, and 22B on skimmilk was analyzed electrophoretically, none was found to be similar to rennet in action. Electrophoretic patterns of skimmilk treated with these

isolates can be seen in Plate IV. The pattern of isolate 16 showed strong proteolysis toward all casein fractions, while the patterns of isolates 17 and 19 closely resembled the control skimmilk. Isolates 17 and 19 exhibited very low enzyme activities, and electrophoretic patterns indicated only slight nonspecific proteolysis on skimmilk. The skimmilk patterns of isolates 22A and 22B showed extensive breakdown of β-casein. Also evident were diffuse bands near the termini--probably degradation products.

It was evident from the electrophoresis study that some of the bacterial enzymes were more suitable as rennet substitutes than others. The enzymes of isolates 1, 2, 16, 22A, and 22B were obviously unsuitable because of extensive proteolysis toward all casein fractions, as shown in the electrophoretic patterns. Proteolysis by the enzymes of these cultures, while nonspecific in nature, markedly altered β-casein. Furthermore, isolates 5, 6, 17, and 19 were eliminated from further consideration as possible rennet substitutes. They exhibited low enzyme activities and the electrophoretic patterns of skimmilk for these isolates were similar to the control skimmilk.

Densitometer analysis. To further evaluate the extent of proteolysis of the skimmilk by bacterial enzymes, electrophoresis gel patterns were scanned using a Photovolt densitometer. The β-casein component was measured using a semiquantitative scale ranging from minus 5 to plus 5 as outlined in "Experimental Procedures". Changes in the β-casein peak were assessed using the control skimmilk scan. Table 5 shows values for skimmilk samples treated with bacterial enzyme preparations of the 15 isolates for 24, 48, and 72 hours of incubation. Since rennet action showed little to no alteration of the β-casein fraction, a densitometer scan value of zero was assigned.

Table 5. Densitometer scan values of the β-casein component when skimmilk was treated with the enzyme preparations of 24, 48, and 72 hours of incubation of the bacterial isolates.

	Scan Values[a]		
	Incubation period at 30 C		
Isolate no.	24 hours	48 hours	72 hours
1	+1.0[b]	0[c]	-5.0
2	-1.5	0	-1.0
3	-2.5	0	-2.5
4A	+1.5	+2.0	-3.5
4B	-2.0	-3.0	-3.5
5		0	-2.0
6		0	0
9A		-2.0	-1.5
9B		-5.0	-1.0
11		+2.0	0
16	-5.0	-5.0	-4.0
17	0	0	0
19	0	0	0
22A	-5.0	-5.0	-5.0
22B	-4.5	-5.0	-4.0

[a]A semiquantitative scale ranging from minus 5 to plus 5.

[b]Positive values represent progressive increase of the β-casein peak.

[c]No alteration of the β-casein peak.

[d]Negative values represent progressive diminution of the β-casein peak.

The scan values of the bacterial enzymes ranged from plus 1 to minus 5 for the β-casein peak. Generally, values for the bacterial enzymes supported the milk-clotting and total proteolytic activity data, as well as

the visual electrophoresis analysis. It was evident that isolates 1, 4A, 4B, 16, 22A and 22B extensively proteolyzed β-casein with values ranging from minus 3.5 to minus 5.0 for the 72 hour enzyme preparations. Isolates 2, 5, 6, 17, and 19, having exhibited low enzyme activities, showed little alteration of the β-casein component. Enzymic proteolysis by isolates 3, 9A, 9B, and 11 on β-casein was lowest when compared to other active organisms. Scan values ranged from zero to minue 2.5. These cultures, therefore, were selected for further evaluation as possible rennet substitutes.

The densitometer scan of the enzymic action on skimmilk for isolate 4A, as an example of the strongly proteolytic enzymes, is shown in Plate V. When compared to the controls and rennet treated skimmilk, β-casein was 80% destroyed. Also shown in Plate V is the densitometer scan of the enzymic action on skimmilk for isolate 19, representing the low activity group.

Selection of one bacterial isolate. The average densitometer scan values of β-casein for three trials when skimmilk was treated with the 24, 48, and 72 hour enzyme preparations for cultures 3, 9A, 9B, and 11 are presented in table 6.

Table 6. Densitometer scan values of the β-casein component when skimmilk was treated with the enzyme preparations of 24, 48, and 72 hours of incubation of isolates 3, 9A, 9B, and 11.

	Scan Values[a]		
	Incubation period at 30 C		
Isolate no.	24 hours	48 hours	72 hours
3	-2.2[b]	-0.6	-2.3
9A	-2.0	-2.3	-2.3
9B	-0.6	-2.6	-2.5
11	-2.0	-1.0	-1.0

[a]A semiquantitative scale ranging from minus 5 to plus 5.

[b]Negative values represent progressive diminution of the β-casein peak.

It can be seen that the scan values for the four isolates were nearly the same at the three time intervals. Isolate 11 exhibited the lowest enzymic action on β-casein--about 20% degraded. The greatest decomposition of β-casein was exhibited by isolate 9B, in which the peak was reduced by 50%.

The actual densitometer scans of the four isolates are presented in Plate VI. The relative amounts of breakdown can be seen for both α_s and β-casein when compared to the skimmilk control and rennet treated skimmilk. Also evident on the scans were numerous small peaks resulting from protein breakdown. The α_s-casein component in these scans showed only slight alterations.

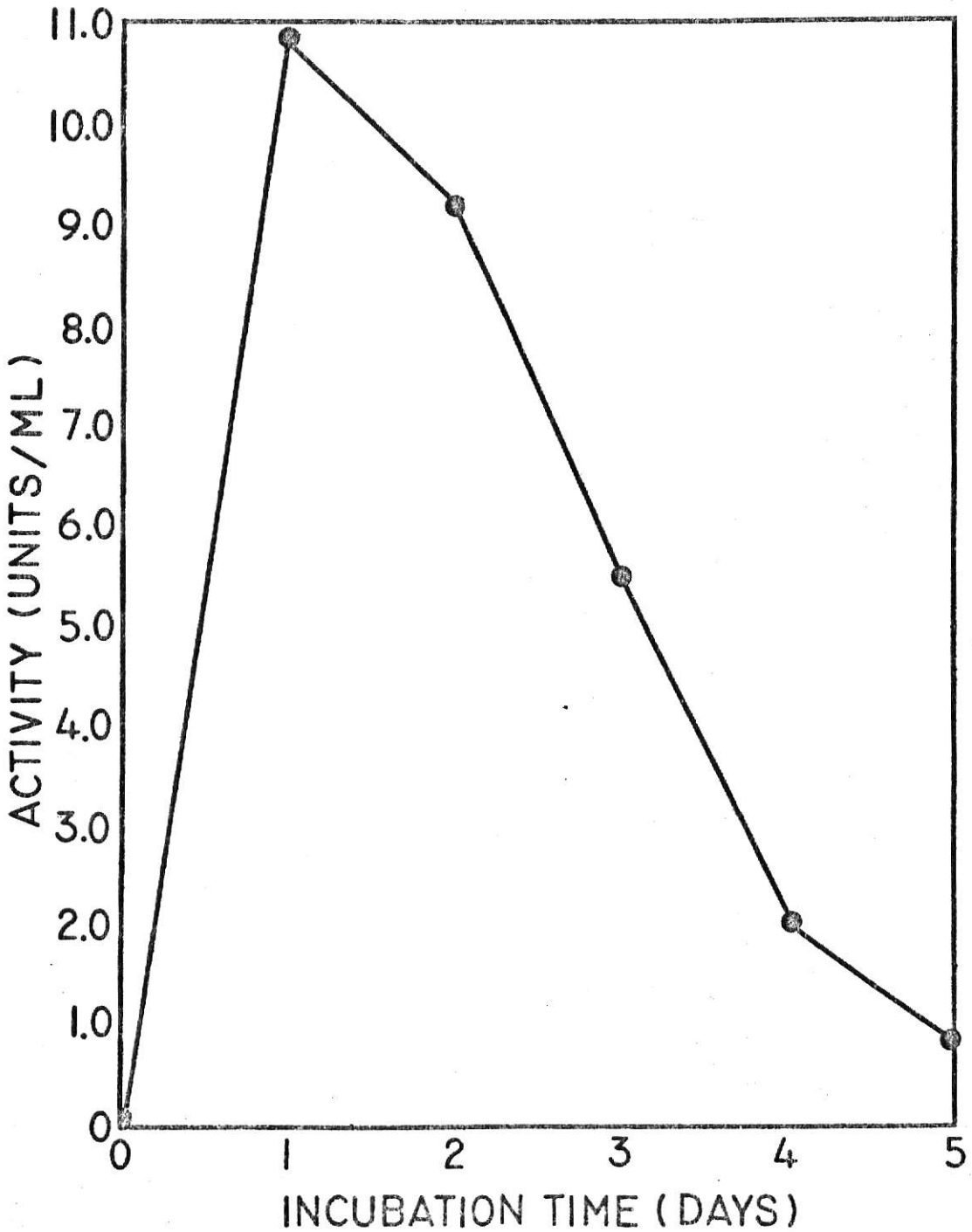


Figure 5. Milk-clotting activity of the Pseudomonas culture grown in 3 l of wheat bran medium in a Brunswick fermentor at 32 C.

The data obtained from the densitometer scans of the enzymic action of isolates 3, 9A, 9B, and 11 were combined with data on milk-clotting activity, total proteolytic activity, and casein-agar gel assays to select one organism for quantitative enzyme production.

Plate VII shows the patterns of isolates 3, 9A, 9B, and 11 on casein agar gel plates. Samples, incubated for 24 hours at 30 C, were taken from the 72 hour old enzyme preparations. All of the isolates exhibited more activity than the 1:2000 dilution of rennet extract shown at the left in each plate. Generally, the bacterial enzymes produced an outer heavy precipitate zone and a smaller inner precipitation zone. The precipitation zones were separated by a clear zone of proteolysis. Generally, the casein-agar gells gave an assessment of the proteolytic nature of the bacterial enzymes.

Results of enzyme activities averaged over three trials for the 24, 48, and 72 hour old enzyme preparations are presented in table 7. Initially, isolate 11 was selected from these 4 isolates for further work. It exhibited the highest milk-clotting activity and the least proteolysis of β-casein. However, this isolate did not respond when grown in a fermentation jar for enzyme elaboration. Isolate 9B showed the highest milk-clotting activity and a nonspecific proteolytic action on the casein fractions. The proteolytic activity of isolate 9A was the lowest of the four organisms studied. However, milk-clotting activity was also low. Therefore, isolate 3 was considered the best culture for continued enzyme studies as the source of a possible rennet substitute.

Table 7. Milk-clotting and total proteolytic enzyme activities for isolates 3, 9A, 9B, and 11 averaged over three trials. Isolates were grown in wheat bran medium at 30 C for 72 hours.

	Incubation period					
	24 hours		48 hours		72 hours	
Isolate no.	MCA[a]	TPA[b]	MCA	TPA	MCA	TPA
3	.263	3.23	.716	5.65	.716	4.82
9A	.215	1.54	.404	3.43	.464	4.29
9B	.756	6.06	1.492	8.04	1.424	8.28
11	NC	0.04	1.030	5.16	2.487	8.91

[a]Milk-clotting activity expressed in units per ml of broth.

[b]Total proteolytic activity expressed in units per ml of broth.

Studies on the Selected Culture

Effect of calcium phytate. Although phytate (inositol hexaphosphate) is naturally present in wheat bran, in this study, wheat bran medium was supplemented with additional amounts of calcium phytate; and its effect on milk-clotting enzyme production was determined.

The results of the influence of calcium phytate on enzyme activity for isolate 3 can be seen in table 8. Calcium phytate when supplemented in wheat bran medium had little effect on milk-clotting enzyme production.

Furthermore, at the higher levels, there was a negative effect on enzyme production. Except for the 1% level of calcium phytate, milk-clotting activity was lower than the control. The results indicated that phytate inherently present in the wheat bran was suffiecent for normal enzyme elaboration for isolate 3.

The total proteolytic activity for isolate 3 was progressively lower for each level of calcium phytate than for the control. Other trials confirmed these results. It was speculated that supplementing wheat bran medium with calcium phytate could be important for suppressing proteolytic enzyme production. Calcium phytate could be useful for milk-clotting enzyme production if a level was chosen in which milk-clotting and total proteolytic enzyme activities were optimum. However, no results were obtained on this hypothesis.

Table 8. The effect of calcium phytate on milk-clotting and total proteolytic enzyme activities of isolate 3, when supplemented in wheat bran (medium no. 5). The isolate was incubated at 30 C under submerged conditions.

	Level of calcium phytate[a]							
	0%		1%		2%		4%	
Incubation period	MCA[b]	TPA[c]	MCA	TPA	MCA	TPA	MCA	TPA
24	.566	1.61	.508	1.74	.473	1.52	.302	0.71
48	.785	2.84	.598	2.22	.561	1.55	.377	0.76
72	.840	3.98	.673	1.04	.615	1.81	.395	1.40
96	.798	4.01	1.364	2.37	.659	2.01	.441	0.94
120	.880	6.63	.777	4.01	.630	3.87	.450	2.22

[a]Expressed in percentage of volume of the medium.

[b]Milk-clotting activity expressed in units per ml of broth.

[c]Total proteolytic activity expressed in units per ml of broth.

Effect of calcium. The effect of calcium from three different sources, including calcium phytate, on milk-clotting and total proteolytic activities was studied. Results of enzyme activities for up to 120 hours of incubation at 30 C can be seen in table 9.

The results confirmed that calcium phytate and not calcium ions was stimulatory to milk-clotting enzyme production. The effect of calcium phytate on milk-clotting activity was somewhat greater in this trial than in the one previously reported. There was a general increase in milk-

clotting activity of the calcium phytate supplemented medium over the control for the entire incubation period. Calcium chloride and calcium phosphate actually decreased both milk-clotting and total proteolytic activities; whereas, calcium phytate enhanced milk-clotting activity and suppressed total proteolytic activity.

Table 9. Effect of calcium on milk-clotting and total proteolytic activity of isolate 3. Calcium was supplemented in wheat bran (medium no. 5). Incubation was carried out at 30 C under submerged conditions.

	Source of calcium[a]							
	Control[b]		Calcium chloride		Calcium phosphate		Calcium phytate	
Incubation period (hours)	MCA[c]	TPA[d]	MCA	TPA	MCA	TPA	MCA	TPA
48	.166	3.01	NC	1.09	.072	0.65	.222	2.06
72	.388	2.02	NC	0.46	.045	0.61	.465	1.24
96	.400	3.72	NC	0.67	.034	0.76	.481	1.68
120	.435	1.77	NC	0.58	NC	1.32	.714	1.72

[a]Calcium ions at the 6% level (w/v).

[b]Wheat bran medium with no added calcium.

[c]Milk-clotting activity expressed in units per ml of broth.

[d]Total proteolytic activity expressed in units per ml of broth.

Effect of pH. Isolate 3 was incubated in wheat bran medium buffered at different pH values. Maximum milk-clotting activity was obtained at pH 6.6 as seen in table 10. The milk-clotting enzyme was not active below pH 6.2. The average milk-clotting activity at pH 7.2 was one-half the average value at pH 6.6. Total proteolytic activity also was highest at pH 6.6. Generally, however, proteolytic activity was affected less than milk-clotting activity by pH, except at pH 5.2.

Effect of temperature. To ascertain the optimum temperature of enzyme elaboration, the selected culture was grown in wheat bran medium at various temperatures. Table 11 presents milk-clotting and total proteolytic enzyme activities for five days of incubation at 25, 30, 37, and 45 C. It can be seen that the optimum temperature for milk-clotting enzyme elaboration was 30 C. This was in agreement with results of the litmus milk temperature study reported previously.

Table 10. Effect of pH of growth medium on milk-clotting and total proteolytic enzyme activities. Isolate 3 grown in wheat bran (medium no. 5) at 30 C under submerged conditions.

	pH of growth medium							
	pH 5.2		pH 6.2		pH 6.6		pH 7.2	
Incubation period (hours)	MCA[a]	TPA[b]	MCA	TPA	MCA	TPA	MCA	TPA
24	NC	0.41	.255	2.15	.588	4.17	.418	3.64
48	NC	0.06	.313	1.74	.811	4.17	.529	3.25
72	NC	0.06	.323	3.57	.806	4.53	.542	4.35
144	NC	0.06	.569	2.52	.906	5.81	.390	3.40

[a]Milk-clotting activity expressed in units per ml of broth.

[b]Total proteolytic activity expressed in units per ml of broth.

Table 11. Effect of temperature of incubation on milk-clotting and total proteolytic activities when isolate 3 was grown in wheat bran (medium no. 5) under submerged conditions.

	Temperature of incubation							
	25 C		30 C		37 C		45 C	
Incubation period (hours)	MCA[a]	TPA[b]	MCA	TPA	MCA	TPA	MCA	TPA
24	.121	2.70	.144	2.66	NC	3.40	NC	0.0
48	.129	2.77	.280	3.44	.103	4.65	NC	0.0
72	.134	3.73	.435	4.38	.220	5.45	NC	0.0
96	.140	3.80	.526	5.16	.351	6.02	NC	1.84
120	.153	3.95	.889	6.20	.395	7.10	NC	2.64

[a]Milk-clotting activity expressed in units per ml of broth.

[b]Total proteolytic activity expressed in units per ml of broth.

Below 30 C, the organism grew slowly and enzyme activities were low. At 45 C, no milk-clotting activity was observed during the five days; and total proteolytic activity was evident only after the fourth day of incubation.

Effect of age of the bacterial culture. The age of the inoculum for maximum milk-clotting enzyme production was determined for isolate 3. It can be seen in Table 13 that the 24 hour old inoculum exhibited the highest milk-clotting and total proteolytic activities. Milk-clotting activity

steadily declined for the 96 hour old inoculum over the five days of incubation, while the 24 hour old inoculum showed a slight increase in activity over the five days. Standard Plate Counts also were higher for the young inoculum.

The 24 and 96 hour old inoculi also were ground with powered alumina and introduced into the growth medium. The broths were then assayed for enzyme activities. Neither culture exhibited milk-clotting activity, and the total proteolytic activity was very low.

It was concluded that isolate 3 elaborated milk-clotting enzymes more readily in the early stage of the growth cycle. Proteolytic enzyme activity was less affected by the age of the inoculum. The fact that the youngest inoculum exhibited the highest enzyme activities, and no activity after the cells were disrupted, indicated that the enzyme was extracellular.

Table 12. Effect of age of the bacterial culture when 24 and 96 hour old inoculi were grown in wheat bran (medium no. 5) at 30 C under submerged conditions.

Incubation period (hours)	Age of inoculum: 24 hour MCA[a]	24 hour TPA[b]	24 hour SPC[c]	96 hour MCA	96 hour TPA	96 hour SPC
24	.333	4.38	8.35	.179	2.90	8.30
48	.321	4.48	7.00	.200	3.16	7.00
72	.204	3.98	7.09	.184	3.98	7.01
96	.323	14.88	8.70	.181	8.94	6.65
120	.450	18.87	9.32	.169	13.72	6.40

[a]Milk-clotting activity expressed in units per ml of broth.

[b]Total proteolytic activity expressed in units per ml of broth.

[c]Standard Plate Count expressed in $\log_{10}$.

Tentative Identification of the Selected Culture

The selected organism was a gram variable nonsporeforming rod, appearing as raised, smooth, and glistening colonies on agar plates. The colonies were rather small at three days, becoming larger at one week and turning slightly brown. The organism was considered nonmotile, exhibiting only a vibrating motion. The culture produced moderately rapid proteolysis of litmus milk and a putrid odor. Optimum temperature for growth of the organism was 37 C. The culture did not survive at 62.5 C for 10 minutes.

Based on the above characteristics, and descriptions by Bergey (15), the organism was tentatively placed in the genus Pseudomonas. However, the combination of characteristics did not conform to any species listed.

Isolation of the Milk-Clotting Enzyme

Enzyme production. The selected Pseudomonas culture was grown in 3ℓ quantities of wheat bran medium for enzyme elaboration. The apparatus used for enzyme production was a specially designed Brunswick fermentor operated in a heated water bath.

In initial trials, attention was given to sterilization techniques of the growth medium, pH control, agitation, and aeration. Because of the large amount of wheat bran used and its insulating effect, a longer autoclaving time was necessary. Maximim agitation and aeration were required for enzyme elaboration.

It was determined that maximum enzyme production was obtained after 24 hours of incubation at 32 C. Figure 5 shows that milk-clotting activity steadily declined after one day of incubation. Activities, representing the average of three different dilution of the enzyme preparations, were higher than any others previously found. Total proteolytic activity also was maximum at 24 hours of incubation. It followed a pattern similar to milk-clotting activity, reaching a high of 87.65 units per ml of broth.

It was necessary to maintain the pH of the growth medium between 6.4 and 6.8. Above 6.8, the milk-clotting enzyme was progressively less active. The enzyme was completely inactive above pH 7.2. When the pH of

the growth medium was adjusted to 6.6, some milk-clotting activity was restored. Proteolytic enzyme activity was less affected by pH. Some proteolytic activity was exhibited at pH 8.0 but not at pH 8.5.

Enzyme concentration. After 24 hours of incubation, the enzyme-rich broth was concentrated by flash evaporation as described in "Experimental Procedures". In preliminary trials, the enzyme-rich broth was flash evaporated in one step to 10% the original volume. However, this technique caused precipitation of the buffer salts and loss of enzyme activity. The enzyme-rich broth, therefore, was flash evaporated in two steps. The final evaporation was made after the buffer salts were removed by dialysis.

Table 13 illustrates the results of a typical concentration procedure. The specific activity of the milk-clotting enzyme was increased in each step of concentration, except for the final flash evaporation step. The lyophilized enzyme showed an over-all four-fold increase in specific activity. The yield of the final product was approximately 38%. It should be noted that this procedure was not aimed at purifying the milk-clotting enzyme, but rather to concentrate the enzyme for future fractionation and study.

Although not shown in the table, the milk-clotting activity of the bacterial enzyme was bout 50% the activity of commercial rennet extract when expressed in R.U./ml. This calculation, made on the lyophilized enzyme, was based on the activity of a 1:5000 (w/w) dilution and activity was determined at 40 C. Milk-clotting activity of the lyophilized enzyme was 1.04 units per mg.

Table 13. Concentration of the milk-clotting enzyme of the selected Pseudomonas organism.

Preparation	Vol. (ml)	Conc. (units/ml)	Total units	Nitrogen (mg/ml)	Specific activity[a] (units/mg)	Yield %	Purification
Wheat bran medium	2560	10.14	25,958	1.43	7.09	100	1
Centrification (supernatant)	2100	10.86	22,806	0.95	11.43	87.8	1.61
Flash evaporation to 50% of original vol.	1025	22.35	22,909	1.90	11.76	88.2	1.66
Dialysate	1325	9.41	12,468	0.32	29.41	48.0	4.15
Flash evaporation to 10% of original vol.	310	26.40	8,184	1.14	23.16	31.5	3.27
	(mg)	(units/mg)	units	(mg/mg)	(units/mg)	%	
Lyophilized product	9530	1.04	9,911	0.037	28.11	38.1	3.96

[a]Specific activity = Concentration (units/ml or units/mg) ÷ Nitrogen (mg/ml or mg/mg).

The total proteolytic activity of the bacterial enzyme increased in each step of concentration. Activity was 276 units per ml of broth after flash evaporation to 10% of the original volume. The total proteolytic activity was almost twice the total proteolytic activity of commercial rennet extract.

It was concluded from results of the concentration procedures that the milk-clotting enzyme of the Pseudomonas culture showed good promise as a possible rennet substitute. It appeared that the limiting factor for its successful use in commercial cheesemaking operations would be the large amount of proteolytic enzyme also present in the lypholized product.

In future studies, work should be directed toward separating the milk-clotting and proteolytic enzyme moieties, and further purifying the milk-clotting enzyme by various fractionation procedures. In assessing the ultimate suitability of the bacterial enzyme as a rennet substitute, the milk-clotting enzyme should be studied in actual cheesemaking trials.

SUMMARY AND CONCLUSIONS

The investigation was directed toward the screening of bacteria for the ability to produce milk-clotting enzymes in an effort to develop a rennet substitute of bacterial origin.

In studying the optimum temperature for growth, it was found that all isolates grew at 30 C. Wheat bran medium was found to be the most nearly suitable for bacterial growth and enzyme elaboration. Submerged conditions of high aeration and agitation were required for maximum enzyme production.

Four cultures whose milk-clotting enzyme showed the most potential as possible rennet substitutes were initially selected from 15 isolates studied for further evaluation. Isolates 3, 9A, 9B, and 11 were categorized into a medium milk-clotting activity group. Electrophoresis analysis showed that the enzymic action on β-casein of skimmilk by these isolates was lowest of all the isolates studied. Densitometer scans showed that β-casein of skimmilk, on the average, was reduced by 40%. The isolates also exhibited mild proteolytic enzyme activity, and produced one precititation zone and one proteolysis zone on casein-agar gel plates. Isolate 3 was considered the best culture for continued enzyme studies as the source of a possible rennet substitute.

Studies on isolate 3 showed that calcium phytate supplemented in wheat bran was only slightly stimulatory for milk-clotting enzyme production. However, calcium phytate did suppress total proteolytic enzyme activity. Calcium was not stimulatory to milk-clotting enzyme production.

In some cases, calcium actually had a negative effect on enzyme production. Maximum milk-clotting activity for isolate 3 was obtained at pH 6.6. The optimum temperature for milk-clotting enzyme elaboration was 30 C. It was also found that isolate 3 elaborated milk-clotting enzymes more readily in the early stage of the growth cycle. The elaborated enzyme was considered to be extracellular.

Isolate 3 was tentatively identified and placed in the *genue* Pseudomonas. However, the combination of characteristics did not conform to any species listed.

For enzyme production and isolation, it was determined that maximum milk-clotting activity was obtained after 24 hours of incubation at 32 C. The culture was grown in 3 - 1 quantities of wheat bran medium in a Brunswich fermentor. Concentration of the milk-clotting enzyme by flash evaporation and lyophilization resulted in a four-fold increase in specific activity and an over-all yield of 38%. The lyophilized milk-clotting enzyme was ab out 50% as strong as commercial rennet extract.

Total proteolytic activity of the concentrated product was about twice the activity of commercial rennet extract. The high proteolytic activity would probably be the limiting factor for the successful use of the bacterial enzyme for cheesemaking.

In future investigations, an attempt should be made to further fractionate and purify the milk-clotting enzyme. The final test for its suitability as a rennet substitute, would be cheesemaking trials.

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SCREENING FOR COAGULATING ENZYMES FROM BACTERIAL SOURCES

by

JOHN WARREN TONEY

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The purpose of this study was to: (a) screen possible bacterial sources for a rennet-like enzyme, (b) study and evaluate the enzymes elaborated, and (c) select one bacterial strain for quantitative milk-clotting enzyme production, and concentration of the elaborated enzyme. The need for developing a substitute for calf rennet, which is in acutely short supply, was the major reason for undertaking this investigation.

Fifteen cultures of unidentified bacteria, isolated from the air, were screened for rennet-like enzymes. All isolates, while showing a wide range of growth temperatures, grew at 30 C in litmus milk cultures.

Reconstituted nonfat dry milk was unsatisfactory as a growth medium for milk-clotting enzyme production. It was useful, however, for determining which bacterial strains elaborated milk-clotting enzymes. Of several other media studied, only wheat bran medium gave satisfactory results. Submerged conditions of high aeration and agitation were required for maximum milk-clotting enzyme production. The milk-clotting activity of a culture grown in wheat bran medium was 100 times higher than when the culture was grown in other media.

The isolates grown in wheat bran medium were categorized into low, medium, and high milk-clotting activity groups. Isolates of the low group exhibited little to no activity. Milk-clotting activities of the medium and high groups were twice and six times higher than activities of the low group, respectively. Milk-clotting activity was defined as the time required for clotting to occur when 2 ml of enzyme-containing broth of a suitable dilution were added to 10 ml of reconstituted nonfat dry milk at 40 C.

Enzymes of the high group were highly proteolytic; whereas, milk-clotting enzymes of the medium group showed the most potential as possible rennet substitutes.

On casein-agar gel plates, all bacterial enzymes produced one precipitation zone and one proteolysis zone. They differed only in the size of the zones produced.

The proteolytic activity of the bacterial isolates ranged from no activity to 17 units per ml of broth. Total proteolytic activity was a measure of the tryosine-tryptophan content in the trichloroacetic acid filtrate of a casein substrate treated with the bacterial enzymes for 30 minutes at 37 C.

Electrophoresis analysis of the bacterial enzymic action on skimmilk showed that the β-casein degradation was lowest for isolates of the medium milk-clotting activity group. Densitometric scans of the electrophoretic patterns showed that the β-casein component, on the average, was reduced by 40%.

Four isolates of the medium milk-clotting activity group were selected for additional evaluation of the milk-clotting enzymes. Based on a combination of results from milk-clotting activity, total proteolytic activity, casein-agar gel assay, and electrophoresis and densitometer analyses, isolate 3 was considered the best culture as the source of a possible rennet substitute. Isolate 3 was tentatively identified and placed in the genus Pseudomonas. The combination of characteristics, however, did not conform to any classified species.

Studies with isolate 3 showed that calcium phytate supplemented in wheat bran medium was only slightly stimulatory to milk-clotting enzyme production. However, it did suppress proteolytic activity. Calcium was not stimulatory to milk-clotting enzyme production. Maximum milk-clotting activity for isolate 3 was obtained at pH 6.6 and 30 C. The milk-clotting enzyme of isolate 3, considered to be extracellular, was most active in the early stage of growth.

For enzyme production and isolation, it was determined that milk-clotting activity was maximum at 24 hours and 32 C. The specific activity of the milk-clotting enzyme was increased four-fold by flash evaporation and lypholization. The over-all yield of the lypholized milk-clotting enzyme was 38%, and was about 50% as strong as commercial rennet extract. The total proteolytic activity was about twice the activity of rennet extract.