Purification, characterization and utilization of bacterial rennet from Bacillus subtilis K-26 for cheese manufacture
DISCUSSION

1. PURIFICATION OF MILK CLOTTING ENZYME

*Bacillus subtilis* K-25 milk clotting enzyme was purified by carrying out a combination of purification procedures involving gel filtration and ion-exchange chromatography (Table 1, Fig.1 to 3).

During the course of the preliminary purification trials of *B. subtilis* K-25 enzyme, a significant loss in the quantity of crude enzyme was observed during dialysis, thereby suggesting the presence of a membrane weakening factor in the crude enzyme. By passing the crude enzyme through Sephadex G-25, 3.9-fold purification with 116% recovery of enzyme activity was achieved (Fig.1). The above technique has also been claimed by Ganchev et al. (1974) to be the best for purification of *Bacillus megaterium* milk clotting enzyme.

The pooled fractions of *B. subtilis* K-25 enzyme obtained after passing through Sephadex G-25 were concentrated with acetone and passed through a Sephadex G-100 column. A 7.6-fold purification with 106% recovery of enzyme activity was obtained (Table 1 and Fig.3).

Sephadex G-100 gel filtration technique for the purification of milk clotting enzymes from different sources has already been used earlier by several workers (Kamimoto et al., 1967; Arima et al., 1968; Ivanov et al., 1976).
The pooled fractions of milk clotting enzyme obtained after passing through Sephadex G-100 was further purified through a column of DEAE-Sephadex A-50, yielding 80% recovery of enzyme activity and 24-fold purification was achieved (Table 1, Fig.3). A similar purification procedure had been adopted by Puhar (1969) for separation of the enzyme complex from *B. subtilis*. Islam and Blanshard (1973) purified bacterial milk clotting enzyme from *Bacillus cereus* using above technique and a 9-fold increase in specific activity with 84% yield was obtained.

It is interesting to note that in the present study, a greater degree of purification (24-fold) of *B. subtilis* K-36 milk clotting enzyme has been obtained with 80% recovery, as compared to many other milk clotting enzymes which have been purified.

II. HOMOGENEITY OF MILK CLOTTING ENZYME

Purified *B. subtilis* K-36 enzyme exhibited a single band moving towards the anode by SDS polyacrylamide electrophoresis (Fig.4), thereby indicating homogeneity of the preparation.

Ultracentrifugal analysis of purified *B. subtilis* K-36 enzyme confirmed that this enzyme has essentially a single homogeneous protein as evidenced by the appearance of a single symmetric peak (Fig.5), thereby resembling other microbial milk clotting enzymes such as *Mucor mucedo* F-27 (Iwasaki *et al.*, 1967), *Mucor rennin* (Arima *et al.*, 1968)
and Lucor mebel (Ottensen and Hickert, 1970a).

The homogeneity of \textit{L. subtilis} K-26 milk clotting enzyme has been further confirmed by isoelectric focussing technique (Fig. 6). The isoelectric point of the purified \textit{L. subtilis} K-26 enzyme was at pH 4.6. The results of the present studies on this enzyme showing one isoelectric point can be compared with the findings of other workers like Foltmann (1966), Iwaseki et al. (1966), Hageneyer et al. (1968), Sardinas (1968), Ottensen and Hickert (1970b), de Koning and Braidma (1973) who studied milk clotting systems like calf rennet, sour rennet, sure-card, Rennilase, Rapidase and Karzyme which had isoelectric points at pH 4.3, 3.5 - 3.8, 4.6 - 5.5, 4.2, 4.58 and 4.58, respectively.

III. PROPERTIES OF PURIFIED \textit{L. subtilis} K-26 MILK
CLOTTING ENZYME

The effect of pH on activity of purified \textit{L. subtilis} K-26 enzyme has been illustrated in Fig. 9. The optimum pH for activity of this enzyme was 7.5. Extracellular protease obtained from \textit{S. faecalis} var. \textit{liquefaciens} has been shown to have an optimum activity at pH 7.4 (Somkuti and Babel, 1966). Gode Chaudhuri et al. (1963) found that milk clotting enzyme produced by \textit{S. faecalis} var. \textit{liquefaciens} showed activity and stability at a pH range between 4.0 and 9.0. The milk clotting activity of \textit{L. subtilis} K-11 was maximum at pH 6.0 (Dutta et al., 1971). The optimum pH for milk clotting activity of \textit{L. curvus} enzyme was at 8.0 (Islam and Blanshard, 1973).
The optimum temperature for maximum activity of purified *B. subtilis* K-36 milk clotting enzyme was 60°C (Fig. 10). Similar type of results have been obtained by Dutta *et al.* (1971) in the case of *B. subtilis* K-11 enzyme. Nalashouris and Tuckey (1967, 1968) showed that maximum milk clotting activity was found at 75-80°C in the case of *B. cereus* enzyme, in contrast to 40 to 45°C reported for calf rennet. Tsuda *et al.* (1964) and Iwasaki *et al.* (1967) found maximum milk clotting activity of *Mucor pusillus* and calf rennet at 56°C and 44°C, respectively. Results of Yu *et al.* (1969a) showed maximum milk clotting activity of *M. pusillus* enzyme at 70°C. Shovers and Davisottt (1967) observed peak milk clotting activity of *E. parasitica* rennet at 57.5°C, while that of calf rennet at 55°C. Sardinas (1972), on the other hand, reported that most of the microbial milk clotting enzymes had maximum activity at 62 to 63°C.

The Michaelis constant for purified *B. subtilis* K-36 enzyme has been found to be 2.77 mg/ml at pH 7.4 and at 37°C, when k-casein was used as substrate (Fig. 13). Islam and Blanshard (1973) reported that the Km of purified *B. cereus* milk clotting enzyme was 3.34 mg/ml with casein as substrate. El-Sadek *et al.* (1975) found that *B. megaterium* enzyme has a Km value of 0.115 with casein as substrate.

The present results showed *B. subtilis* K-36 milk clotting enzyme was found to be stable at 40°C for 30 minutes, whereas at 60°C, the enzyme retained
only 10% of its original activity (Fig. 11). In the patent that have been filed by some workers, it has been claimed that the enzyme from B. cereus is destroyed within 30 minutes at 70°C (Sardinas, 1972). However, Melachouris and Tuckey (1968) found that the milk clotting enzyme from B. cereus was destroyed within 3 minutes at 65°C. El-Sadek et al. (1974) showed that B. mesenterium milk clotting enzyme was completely inactivated at 55°C or above after 24 hr. Kean et al. (1973) observed that an alkaline protease obtained from B. subtilis N4b B3411 was stable up to 30 minutes at 60°C but was rapidly inactivated at 70°C. Results of Sokutie and Babel (1966) indicated that an extracellular protease obtained from L. caseiia var. liquefaciens was almost completely destroyed by heating at 85°C for 5 minutes. Tsuge et al. (1964) reported that animal rennet lost 35% or more of its activity at 50°C within 60 minutes, while Meito rennet showed a loss in enzyme activity up to 20% only. According to Sardinas (1969), milk clotting enzyme obtained from B. parasiticus was completely inactivated at 60°C within 5 minutes.

Results of the present study on the storage of purified milk clotting enzyme from B. subtilis K-33 in liquid form at different temperatures have shown (Table 5) that loss of enzyme activity was pronounced after storage of the enzyme at 37°C for 10 days, while 100% enzyme activity was retained when the enzyme was stored at -18°C for 30 days. The present findings are in agreement with those of Malik and Nair (1977) on crude B. subtilis K-33 milk clotting enzyme stored in liquid form at -18°C for 7 days. Studies by El-Sadek
et al. (1974) showed that *R. mesenterium* milk clotting enzyme was stable at 10°C or below for 7 days, but was completely inactivated at 50°C or above after 24 hr.

Ottensen and Rickert (1970a) observed that the liquid form of calf rennet lost 40% of activity at 25°C within 2 days, while *H. midei* enzyme remained stable up to 8 days in the pH range of 2.0 to 6.0. Yu et al. (1969b) noticed that *H. musillus* rennet was stable for 15 days at pH 5 at 30°C.

The milk clotting activity of *R. subtilis* K-36 enzyme peaked at 4 \times 10^{-3} M CaCl₂ (Fig. 14). Similar type of results have been reported for *R. parasitica* rennet by other workers (Sardinas, 1968; Shovers and Savisotto, 1967). As compared to calf rennet, the milk clotting activity of *Mucor* rennet is said to be influenced to a greater extent by calcium chloride (Tsugo et al., 1964; Iwasaki et al., 1967; Richardson et al., 1967; Pederson, 1969).

In order to get an insight into the nature of an enzyme, its cofactor requirements and the nature of the active centre, activators and inhibitors are generally employed in enzymatic studies. Such studies also help in comparing the properties of one enzyme with those of others. The present investigation with *R. subtilis* K-36 milk clotting enzyme has given very useful and interesting information in this regard.
The milk clotting activity of *B. subtilis* K-26 enzyme was appreciably inhibited by $10^{-3} M$ concentration of $Mg^{++}$, $Pb^{++}$, $Cu^{++}$ and $Ni^{++}$ while $Ag^{+}$ and $In^{++}$ showed no inhibitory effect on enzyme activity (Table 5).

A report by El-Beick *et al.* (1974) showed that the milk clotting activity of *B. megaterium* enzyme was inhibited by $Cu^{++}$, $Hg^{++}$, $Zn^{++}$, $Ca^{++}$, $Fe^{++}$ and $Li^{++}$ ions as well as by mercaptobenzoxinol, while $Co^{++}$, $Mg^{++}$, $NO_3^-$, $Mn^{++}$ and $NaSO_4$, as well as aspartic acid, EDTA and L-cysteine had no inhibitory effect on the enzyme activity.

The milk clotting enzyme from *B. subtilis* K-26 was completely inhibited by EDTA (Table 6). Similar type of results have been reported in the case of milk clotting enzyme obtained from *B. cereus* by Islam and Blanshard (1973). The irreversible inhibition of the bacterial milk clotting enzyme by EDTA has been explained as due to simultaneous removal of both $Ca^{++}$ and the catalytically important $Zn^{++}$ from the enzyme (Islam and Blanshard, 1973).

Sternberg (1971) reported that the enzyme obtained from *Lucor michaei* was activated by EDTA, $MnCl_2$, $CaCl_2$, $NH_4Cl$ and Diisopropylfluorophosphate.

The inhibition of milk clotting activity of *B. subtilis* K-26 enzyme by $10^{-3} M$ concentration of $KmO_4$ observed in the present study, indicates that oxidizable amino acids may be present in the enzyme in its active site. Studies by Iwasaki *et al.* (1967) showed that *B. subtilis* enzyme was
inhibited by KMnO₄. As a result of studies on the effect of oxidizing agents, photo-oxidation and reaction with Diazotetrazole on enzyme activity, Yu et al. (1970) have concluded that histidine is present in the active centre of the milk clotting enzyme retained from B. subtilis lindt. In order to locate the active centre of B. subtilis K-36 enzyme similar investigations with other specific inhibitors need to be carried out.

Addition of 1,10-phenanthroline to the milk clotting enzyme of B. subtilis K-36 preferentially removed Zn⁺⁺, but not Ca⁺⁺. Since the milk clotting enzyme of B. subtilis K-36 is partially reactivated by the addition of Zn⁺⁺, it seems likely that as with other enzymes, removal of Ca⁺⁺ destroys the structural stability of the enzyme, whereas the Zn⁺⁺ is primarily concerned with enzyme activity (Islam and Dinsmore, 1973).

The fact that milk clotting enzyme from B. subtilis K-36 is not affected by the presence of DFP suggests the possible absence of a serine residue in the active centre of the enzyme. Similar type of results have already been reported by Dutta et al. (1971) in case of milk clotting enzyme produced by B. subtilis K-11.

Bacillus subtilis K-36 enzyme was not inhibited by sulphydryl inhibitors like mercaptoethanol and thiomersal (Table 6). In this respect, B. subtilis K-36 enzyme
appears to be similar to most other milk clotting enzymes in not having any sulphhydryl group in its active centre. The inhibition of activity of \textit{A. subtilis} K-35 enzyme by EDTA and \textit{KCN} as well as the reversible inhibition by \textit{1,10-phenanthroline} suggests that the above enzyme may be a metallo-protein. Atomic absorption analysis of the \textit{A. subtilis} K-35 enzyme has revealed that it contains an average of 1350 \( \mu \text{g} \text{Zn}^{++} \) per gram of protein. In this respect \textit{A. subtilis} K-35 enzyme appears to be similar to the milk clotting enzyme obtained from \textit{A. cereus} (Islam and Blanshard, 1973).

The molecular weight of \textit{A. subtilis} K-35 enzyme as determined by gel filtration technique was 37,000 (Fig. 15). The results of gel filtration chromatography have also suggested that this enzyme has a single homogeneous component.

Considerable variations in molecular weight of milk clotting enzymes from microorganisms have already been recorded in literature (Animal rennin mol. wt. 34,000 - de Koning, 1967; \textit{Bacillus cereus} mol. wt. 50,000 - Islam and Blanshard, 1973; \textit{Endothia parasitica} mol. wt. 34,000 - Barilions, 1963; \textit{Mucor micheii} mol. wt. 30,000 - Sternberg, 1971; \textit{Mucor musillus} mol. wt. 32,500 - Arima et al., 1970). But the results of the present study on molecular weight for \textit{A. subtilis} K-35 enzyme are in good agreement with the findings of Keay et al. (1970) and Keay and Wildi (1970) who obtained a molecular weight of 36,200 in case of a number of alkaline
and neutral proteases from *Bacillus* sp. The alkaline and neutral proteases of *B. subtilis* var. *avloesaccharitius* has also been found to have a molecular weight of 22,700 (Isuru *et al.*, 1967a).

Results on the amino acid analysis of purified *B. subtilis* K-36 enzyme (Table 7) have indicated that there are some similarities in the amino acid composition of the above enzyme and chymosin, except that amino acids such as alanine, half-cysteine, arginine and methionine content are present in higher quantities, while valine, leucine and glutamic acid are in lower quantities in *B. subtilis* K-36 enzyme.

Considerable variations in the amino acid composition of milk clotting enzymes from other microorganisms have been reported in literature (*Mucor mesilium*, Arima *et al.*, 1970; *Mucor miehei*, Ottensen and Rickert, 1970a, b; *E. parasitica*, Whitaker, 1970). The amino acid analysis of purified *B. subtilis* K-36 enzyme reported in the present study, is at variance with the result of Sardinas (1968) who did not find any lysine, methionine or tryptophan in the crystalline enzyme preparation studied by him in the course of two separate assays.

The purified bacterial milk clotting enzyme from *B. subtilis* K-36 preferentially hydrolyzed the casein fractions in the following order: \( \alpha \)-casein, \( \kappa \)-casein, \( \beta \)-casein (Table 8). Similar results have been reported for *Mucor mesilium* enzyme, while for *Endothia parasitica* enzyme, the order of hydrolysis was as: \( \alpha \)-casein, \( \beta \)-casein and \( \kappa \)-casein (Tam and Whitaker, 1972).
IV. CHANGES DURING CHEESE MANUFACTURE

The results presented in Fig. 16 show that acid development was relatively faster in the case of cheese made with crude or purified bacterial milk clotting enzyme when used either singly or in combination with calf rennet or swine pepsin. Similar results have been reported by other workers when commercial rennet substitutes like Milcozyme (Milcozyme Information, 1969) and Remilase were used for cheese manufacture (Janet and Alais, 1973).

Bacterial milk clotting enzymes showed a slightly higher protein loss in whey when either calf rennet or a combination of calf rennet and swine pepsin in a ratio of 50:50 was used for cheese making. This high protein loss may be due to either a higher rate of proteolytic activity or lesser specificity of the enzymes. Similar trends in regard to higher protein losses in cheese whey have already been reported by Singh et al. (1967) in case of crude milk clotting enzyme of R. subtilis, R. mesenterium, and R. aereria. Puhar and Irvine (1973) observed similar protein losses in the case of a protease obtained from a mutant strain of R. subtilis. 'Milcozyme', a commercial rennet substitute from P. polymyxa was also considered unsuitable by Reps et al. (1973) for the production of Cheddar, Tilsit and Kortowski cheeses, presumably due to excessive proteolysis in the curd, thereby resulting lower quality of cheese.

Bacterial milk clotting enzymes showed a slightly higher fat loss in whey than calf rennet. The fat loss in cheese
whey is similar to that found in calf rennet as well as when calf rennet and purified bacterial enzyme were used in 50:50 ratio (Table 9). Higher fat losses in whey have also been reported by earlier workers (Srinivasan et al., 1963b; Singh et al., 1967). The probable causes for higher fat losses in whey in the case of bacterial rennet is due to the lowering of the surface tension of the fat globules by lipase present in the milk clotting enzyme. Fat losses in whey may also be due to breakdown of casein micelle followed subsequently by reduced ability of the enzyme to occlude fat (Puhan and Irvine, 1973). Higher fat losses in cheese whey were also observed by other workers in case of L. casei milk clotting enzyme (Kikuchi et al., 1968c; Antila and Aapola, 1969a, b). However, Zwaginga et al. (1963) did not find any difference in the composition of whey when Lactococcus enzyme and calf rennet were used for cheese manufacture.

The yield of cheese was 11.2% when calf rennet and purified B. subtilis K-35 milk clotting enzyme were used in 50:50 proportion, whereas with the use of crude B. subtilis K-35 milk clotting enzyme alone, the cheese yield was only 10.3% (Table 9). The lower yields of cheese obtained with crude bacterial enzyme may presumably be due to higher lipolytic and proteolytic activities, thereby resulting in greater protein and fat losses in whey. The lower moisture content in bacterial rennet cheese may also be partly responsible for the lower cheese yields.
Similar results have also been reported by other workers who have used the protease from *B. subtilis* in cheese making trials (Srinivasan et al., 1963; Behnke, 1967). During the course of their studies on the manufacture of a Canadian variety of cheese, Puhan and Irvine (1973a) observed that proteolysis was continuous when it was brought about by bacterial protease used either singly or in combination with other enzymes, thereby resulting in the formation of a softer curd with more protein losses in whey. The fat losses in whey were also relatively higher in the experimental cheese lots made with bacterial rennet. Kikuchi et al. (1968b,c) observed higher protein losses in whey when *N. patillus* rennet was used for cheese making. Cheese made with calf rennet showed lesser protein losses in whey. The dry matter and fat losses were also higher with the former enzyme. Although higher proteolytic breakdown had been observed with Meito rennet cheeses, Tsugo et al. (1964) obtained almost similar yields of cheese with Meito and calf rennets. According to the above authors, addition of calcium chloride resulted in better yields of Gouda cheese. Prins (1973) has also recommended addition of CaCl₂ before renneting for obtaining higher cheese yields.

V. CHANGES DURING CHEESE RIPENING

During the six months of ripening period, cheese made with bacterial enzyme had a decreased moisture as compared to cheese made with calf rennet (Table 10), although the
Initial moisture retention in the former case was lesser than in the latter (Fig. 17).

Murray and Gruetmaier (1970) suggested some technological changes during the manufacture of Cheddar cheese made with *B. subtilis* enzyme for moisture retention and firmness in cheese within such limits that are normally obtained in cheese made from calf rennet. The modifications suggested by the above workers refer to the application of heat during the first 30 minutes of the 45 minutes of holding time of milk, followed by a raise in the temperature of curd to 110°F for 3 minutes and thereafter, quick cooling of the curd to the normal clotting temperature of 102-104°F.

There was a gradual rise in pH in cheeses made with all the five milk coagulating systems (Table 11). The results of the present finding indicate that the changes occurred in cheese may be due to the action of the milk clotting enzyme on the primary decomposition products, followed by accumulation of compounds like neutral carbinols, soluble peptides and basic amino acids which contribute to the flavour and taste of the cheese.

The above observations show a similar trend in acid formation and pH as those noticed earlier by Tsugo *et al.* (1964), Kikuchi *et al.* (1968b) and Kikuchi and Toyoda (1969) who used Meito and calf rennets for the preparation of Gouda, Cheddar and Edam cheese varieties. The total nitrogen content in all the five types of rennet cheeses examined in the present study did not show such variation during the six month ripening period, whereas there was a steady increase in soluble nitrogen
content in all the five types of cheese during ripening (Table 13).

Crude bacterial milk clotting enzyme gave rise to higher soluble nitrogen content in cheese than either purified bacterial enzyme or calf rennet or a combination of both the enzymes (Fig. 19). The rate of increase in soluble nitrogen was highest in cheese made with crude bacterial enzyme, followed by a decreased trend in soluble nitrogen in cheese made with purified bacterial enzyme, purified bacterial milk clotting enzyme and calf rennet (50:50), purified bacterial milk clotting enzyme and swine pepsin (50:50) and lastly, calf rennet (Table 13).

The maturity index of cheese was 0.37, 0.55, 0.47, 0.44 and 0.45, respectively (Table 14) in 5 days old cheese made with calf rennet, crude bacterial enzyme, purified bacterial enzyme, mixture of purified bacterial enzyme and calf rennet (50:50), mixture of purified bacterial enzyme and swine pepsin (50:50). After ripening for six months, cheese made with the mixed coagulant (purified bacterial enzyme and swine pepsin, 50:50) showed a lower maturity index as compared to cheese made with crude bacterial enzyme or purified bacterial enzyme (Fig. 19). However, maturity index value in the above cheeses was higher as compared to those found in cheeses made from calf rennet. Mixed coagulant consisting of purified bacterial enzyme and calf rennet in 50:50 proportion, gave slightly higher maturity index as compared to the mixed coagulant consisting of purified bacterial enzyme and swine pepsin or calf rennet (Fig. 19). The above results indicate a
higher proteolytic breakdown initially in bacterial rennet cheese but not in calf rennet cheese. These results are similar to the findings of Kikuchi et al. (1968b,c) in Edam, Gouda and Cheddar cheeses prepared with Meito and calf rennets.

Tsugo et al. (1964) observed lower quantities of water soluble protein nitrogen in Meito rennet cheese than in calf rennet cheese. The reason for this lower value of soluble nitrogen has been attributed by the above workers to release of larger non-casein nitrogen fractions by Meito rennet.

The higher rates of increase in the maturity index in cheese made with bacterial milk clotting enzymes as compared to calf rennet, have also been observed in the case of Meito (Tsugo et al., 1964; Kikuchi and Toyoda, 1969) and in Enothia rennet cheeses (Edelsten et al., 1969). Amongst cheeses made with different types of microbial rennets, Enothia rennet cheese had higher rates of increase in maturity index than Meito or M. miehei rennet cheese. Cheese made with the mixed coagulant consisting of 50:50 combination of purified bacterial milk clotting enzyme and swine pepsin, exhibited lower maturity index as compared to cheese made with either crude or purified bacterial milk clotting enzyme, but maturity index values in these cheeses were definitely higher than those found in calf rennet cheese (Fig. 19).

According to Maragoudakis et al. (1961) and Melachouris and Tuckey (1964) swine pepsin could be used satisfactorily
as a rennet substitute for cheese making, provided the cheese was matured for longer periods than that would be required for calf rennet cheese. Raadsveld (1964) observed that cheese made with pepsin had a somewhat slower ripening in so far as proteolysis was concerned. Among three pepsin preparations that were tried by Raadsveld (1964), two preparations yielded cheeses with a similar or higher flavour scores while the third one gave a significantly lower flavour score. The above studies indicated, however, that when swine pepsin was mixed with bacterial milk clotting enzyme, the resultant cheeses had higher rate of maturity index than calf rennet cheese.

Bacterial milk clotting enzyme as well as calf rennet cheeses exhibited clear cut differences between them in regard to starch gel electrophoretic characteristics (Fig. 2). While bacterial milk clotting enzyme cheese showed degradation of both $\alpha_s$- and $\beta$-casein fractions, in calf rennet cheese only $\alpha_s$-casein was degraded. Thus, bacterial milk clotting enzyme brought about higher proteolytic breakdown in cheese than calf rennet. Nickelson and Fish (1970) have also reported similar differences in the electrophoretic patterns in casein digested with different milk clotting enzymes. According to the above authors, higher proteolytic breakdown of whole casein was noticed with Endothia than with calf or Meito rennets. The former digested $\beta$-casein to a greater extent than other milk clotting enzymes. The results of the present investigation are in agreement
with the earlier findings of Ledford et al. (1968) and Al-Megdomy (1968) on $\alpha$- and $\beta$-casein degradation in cheese.

Organoleptic evaluation studies on six month old cheese indicated that cheese made with mixed coagulant consisting of purified bacterial milk clotting enzyme and calf rennet in 50:50 proportion, was the most acceptable, as compared to cheese made with calf rennet alone (Table 15).

The results of the present study indicate that a combination of purified bacterial milk clotting enzyme and calf rennet in 50:50 proportion can be used satisfactorily as a rennet substitute for cheese manufacture. The above mixed coagulant combination has the added advantage in not being inhibitory towards growth of starter culture during cheese manufacture. The yield of cheese obtained with above mixed coagulant was, however, similar to that of calf rennet.