REVIEW OF LITERATURE

Purification, characterization and utilization of bacterial rennet from Bacillus subtilis R-33 for cheese manufacture
REVIEW OF LITERATURE

1. PURIFICATION OF MICROBIAL MILK CLOTTING ENZYMES

Enzymes which are either in concentrated or dilute form are subjected to purification, generally by fractional precipitation using salts or organic solvents or by applying techniques such as gel filtration, column chromatography and adsorption on ion-exchangers. Homogeneity of the purified preparation is achieved by paper or gel electrophoretic technique at different pH levels either by ultracentrifugal analysis, or by the determination of absorption spectra.

1. Milk clotting enzymes from bacteria

Although a large number of bacteria including aerobic spore forming bacilli have been known to elaborate the milk clotting enzymes, enzyme purification studies have been attempted with only a few such organisms.

1.1. Bacillus cereus

Islam and Blanshard (1973) have demonstrated that the milk clotting proteolytic enzyme from a strain of B. cereus could be isolated and purified from the culture filtrate by acetone or ammonium sulphate fractionation followed by DEAE-cellulose and DEAE-Sephadex A-50 column chromatography. The purification resulted in about 9-fold increase in specific activity of the enzyme with 24% yield. The enzyme was also found to be homogeneous by electrophoresis.
1.2. *Bacillus mesenterium*

The milk clotting enzyme of *B. mesenterium* was purified 39-fold by ammonium sulphate fractionation followed by gel filtration through Sephadex G-150 (El-Sadek et al., 1974). The recovery of the enzyme, however, was only 12%.

1.3. *Bacillus mesentericus*

The milk clotting enzyme present in the culture liquor of *B. mesentericus* was purified by fractionation with different types of Sephadex gels. Sephadex G-25 proved the best in this respect with 0.1M CaCl₂ as an eluent (Ganchev et al., 1974).

Ivanov et al. (1976) purified milk clotting enzyme from *B. mesentericus* by precipitation of the crude extract with ethanol, gel filtration on Sephadex G-100, followed by electrophoresing.

1.4. *Bacillus subtilis*

Tipograf et al. (1966) claimed to have absorbed the milk clotting enzyme produced by *B. subtilis* and *B. mesentericus* on to kieselghur upto 94%. The enzyme was eluted with 0.3M phosphate buffer at pH 6.0.

According to a report by Toyo Shisei Co. (1968), practically all milk clotting activity could be recovered from broth by precipitation with ammonium sulphate.
The enzyme complex from *B. subtilis* has also been purified by Pujah (1969) by sequential chromatographies on columns of CM-cellulose, DEAE-Sephadex A-50 and by re-chromatographing on SE-Sephadex. These proteases were designated as acid, neutral and alkaline types, depending upon their optimum pH range for casein degradation.

The peptide enzyme complex produced by a strain of *B. subtilis* has been shown to contain an acid peptidase and a neutral peptidase but substantially free from any alkaline peptidase activity. The enzyme was shown to have high milk coagulating and low proteolytic activity. A process for the production of the above enzyme complex has also been described (John Labatt Ltd., 1970).

Partial separation of the milk clotting enzyme activity from other proteolytic enzymes of *B. subtilis* was achieved by Kuila et al. (1971) using casein as the adsorbent. A three-fold purification of the milk clotting component from the protease system of *B. subtilis* K-11 has also been reported by ammonium sulphate fractionation (Dutta et al., 1971).

1.5. *Streptococcus faecalis* var. *liquefaciens*

An extracellular proteinase of *S. faecalis* var. *liquefaciens* was isolated from the culture filtrates by precipitation with (NH₄)₂SO₄ and subsequently purified on Sephadex G-75 (Somkuti and Babel, 1963).
2. **Milk clotting enzymes from moulds**

Milk clotting enzymes from a large number of moulds have been extensively purified in recent years by making use of several elegant biochemical techniques.

Sardinas (1966) purified milk clotting enzyme of *Endothia parasitica* using ammonium sulphate precipitation and acetone treatment, followed by filtration through carbon and finally, precipitation with isopropanol.

Hagemeyer et al. (1968) purified the protease obtained from *E. parasitica* by two methods. The first method involved ammonium sulphate fractionation, gel filtration on Sephadex G-100 followed by chromatography on DEAE-cellulose at pH 4.6. In the second method, ammonium sulphate fractionation, acetone fractionation, charcoal treatment, gel filtration on Sephadex G-100 and ion-exchange chromatography on DEAE-cellulose were used. Alais and Novak (1970) were also able to separate the enzyme from *Endothia parasitica* into three fractions by using DEAE-cellulose column chromatography. Milk clotting and proteolytic activities were located in the second fraction which represented 40-45% of the total yield. The enzyme from *E. parasitica* was purified by passing through columns of Sephadex G-75, Sephadex G-200 and CM-cellulose (Sequi et al., 1972).
Mucor miehei rennet has been fractionated into three fractions by gel filtration through Sephadex G-100 column (Edelsten and Jensen, 1970). Ottensen and Rickert (1970) have described a purification procedure of renninase from Mucor miehei using ammonium sulphate fractionation, batch-wise adsorption on DEAE-Sephadex, SE-Sephadex followed by DEAE-Sephadex chromatography and gel filtration. These purification steps finally yielded a homogeneous preparation with 14-fold increase in specific activity of the enzyme.

Sternberg (1972) used ammonium sulphate fractionation and ion-exchange chromatography on Amberlite CG-50 and CM-cellulose columns to purify milk clotting enzyme from Mucor miehei. The recovery was about 37,2%. Rickert and Elliot (1973) utilized a diafiltration cell to obtain a highly purified preparation of acid protease from Mucor miehei, with 80% recovery.

Purification of microbial rennet from the culture filtrate of Mucor miehei using the technique of reversible precipitation of protein with polyacrylic acids has been reported by Sternberg (1976).

Iwasaki et al. (1967) achieved 10,6-fold purification of the milk clotting enzyme from Mucor pusillus Lindt by passing it through columns of Amberlite IRC-50 and DEAE-Sephadex A-25.
Somkuti and Babel (1968) purified the acid protease obtained from *Mucor pusillus* by treatment with ammonium sulphate, ethyl alcohol, gel filtration and ion-exchange chromatography.

Arima *et al.* (1968) obtained a crude preparation of the milk clotting enzyme from *Mucor pusillus* var. Lindt. and purified it by column chromatography using Amberlite CG-50, DEAE-Sephadex A-50 and Sephadex G-100. The purified enzyme was crystallized by saturation to 40% with \((\text{NH}_4)_2\text{SO}_4\) followed by dialysis against a solution of increasing \((\text{NH}_4)_2\text{SO}_4\) concentration. The crystalline enzyme thus obtained was purified to 14.5-fold with 13.1% yield.

Patents have been taken by Organon Laboratories Ltd. (1971) and by Moelker and Matthijsen (1971) in regard to purification of microbial rennets from *Mucor pusillus* Lindt. and *Endothia parasitica*. The respective enzymes were treated with an adsorbing silicate, silicon dioxide, bentonite or zeolite at pH 3.0-9.0 to selectively remove any unspecific proteolytic enzymes responsible for imparting bitterness to cheese.

Partial separation of milk clotting and proteolytic activities were achieved by Morvai-Racz (1974), using fractional precipitation with ethanol, acetone and Amberlite CG-50 ion-exchange chromatography.
II. PROPERTIES OF MICROBIAL MILK CLOTTING ENZYMES

Proteolytic enzymes obtained from different sources are known to exhibit major or minor differences in regard to their properties. A thorough knowledge of their properties is of primary importance to any good cheese maker, if these enzymes are to be used as rennet substitutes, since the quality of the final product is, to a large extent, dependent upon the type of the coagulant used during cheese manufacture. The complexities involved in the manufacturing and ripening processes of cheese offer sufficient scope for introducing modifications in the techniques of manufacture to suit the type of enzyme substitutes used during the cheese manufacturing process.

1. Bacterial milk clotting enzymes

1.1. Bacillus cereus

The milk clotting enzyme produced by B. cereus has been studied by many workers such as Choudhary and Nikolajcik (1970, 1971), Melachouris and Tuckey (1968), and Srinivasan et al. (1962a, b). Miles Laboratory Inc., has obtained an extensive patent coverage on the above coagulant (Sardinas, 1969).

According to patented literature, the enzyme is stated to be destroyed within 20 minutes at 70°C and to maintain its milk curdling potency at pH 4.8 (Sardinas, 1972). Melachouris and Tuckey (1968) found, however, that the above enzyme was destroyed in 3 minutes at 65°C and was less sensitive to pH
changes than calf rennet. The enzyme exhibited maximum rennet activity at 75 to 80°C, in contrast to 40 to 45°C reported for animal rennet. The extent of casein degradation was also greater with this enzyme. The specific proteolytic action of the B. cereus enzyme on \( \beta \)-casein was somewhat similar to that obtained with calf rennin although the former preferentially hydrolysed \( \beta \)-casein than \( \alpha_s \)-casein (Choudhry and Nikolajcik, 1970, 1971).

Islam and Blanshard (1973) have described purification and characterization of the milk clotting enzyme produced by B. cereus strain X-20. The purified enzyme was found to be homogeneous by acrylamide gel electrophoresis at pH 3.5 to 8.6, and had a molecular weight of 50,000. The optimum activity of the above enzyme was at pH 8.0 and at 40°C when casein was used as the substrate. The enzyme was most stable at pH 6.0 and also resisted freezing and freeze-drying. Heavy metal ions inactivated the enzyme. The enzyme activity was inhibited irreversibly by EDTA but reversibly by 1,10-phenanthroline. The above enzyme has been identified as a \( \alpha \)n-containing protease.

1.2. Bacillus megaterium

El-Sadek et al. (1974) showed that milk clotting enzyme produced by B. megaterium was maximally stable at pH 5.0 but lost its activity quite rapidly below pH 5.0 and also above pH 7.0. The enzyme was stable at 0°C or below for 7 days but was completely inactivated at 50°C or above after 24 hr.
The milk clotting activity of the enzyme was inhibited by Cu$^{2+}$, Mg$^{2+}$, Zn$^{2+}$, CH$_3$, Fe$^{2+}$ and Li$^{+}$ ions and mercaptoethanol. On the other hand, Co$^{2+}$, Mg$^{2+}$, NO$_3^-$, Mn$^{2+}$ and NaSO$_4$ ions as well as aspartic acid, EDTA and L-cysteine did not have any inhibitory effect on the activity of B. megaterium enzyme. The activity of the above enzyme was shown to be directly proportional to enzyme concentration over the range of 0.021 to 0.190 mg/ml. The $K_m$ value of the above enzyme with casein as a substrate was 0.114, while the maximum velocity was 0.15 μ mole tyrosine/min/mg protein (El-Sadek et al., 1975).

1.3. *Bacillus polymyxa*

Godo Shusei Co. (1968, 1969) and Imai et al. (1970) found that milk clotting enzyme produced by *B. polymyxa* was a thermostable alkaline protease. The activity and stability of the enzyme ranged between pH 4.0 and 9.0.

1.4. *Bacillus subtilis*

The most extensively studied bacterial rennet is the one elaborated by *B. subtilis*. The organism is known to produce three proteolytic enzymes namely, acid, neutral and alkaline proteases (Murray and Kendall, 1969; Puhon, 1969). The ratio of these three types of enzymes may vary from one strain to another and also with different cultural and environmental conditions. Puhon (1969) reported the presence of neutral protease in largest amounts, while acid protease was the lowest and the alkaline protease occurred in moderate quantities in the enzyme preparation.
According to Murray and Kendall (1969), the milk clotting activity of *B. subtilis* rennet is dependent upon at least one monovalent metal ion (i.e., potassium) and one divalent ion (i.e., magnesium). Puhan (1966, 1968) has studied in detail the production and properties of proteases from *B. subtilis*. The neutral protease was found to be stable at pH ranging from 6.5 to 10.0 and was inhibited by ethylenediaminetetraacetate. The enzyme released 33% more non-protein nitrogen than animal rennet up to milk clotting stage. Alkaline protease, on the other hand, proved to be more stable at a pH range of 5.0 to 10.0 and was inhibited by potato inhibitor. Manifestation of esterase activity was also noted. The enzyme released three times more non-protein nitrogen than animal rennet. The neutral protease digested $\alpha$- and $\beta$-casein non-specifically but rapidly (Puhan, 1969).

Tsuru et al. (1967a, b) have also studied the proteolytic specificities of alkaline and neutral proteases of *B. subtilis* var. amyloliquefacientis. The molecular weight and sedimentation coefficient were estimated as 22,700 and 2.805, respectively, while the terminal amino acid has been identified as alanine. The neutral protease split the $\beta$-chain of oxidized insulin at eleven sites of the peptide linkages, thereby indicating its narrow specificity as compared to subtilopectidase A. The above results also indicate that with a few exceptions, the peptide bonds susceptible to the action of the neutral protease were mainly those involving amino group of hydrophobic amino acids and tyrosine. *Bacillus subtilis* protease showed potent casein digestion at neutral pH and milk clotting at pH 5.6,
whereas the enzyme was not at all active on esters and keratin and only slightly active towards elastin.

Dutta et al. (1971) reported that the milk clotting and proteolytic activities of *B. subtilis* K-11 were maximum at 60°C, at pH 6.0 and 8.0, respectively. Trypsin inhibitors had little effect on either of the activities, while \(10^{-2}\)M cysteine completely inhibited both types of activity. EDTA inhibited proteolytic activity completely but milk clotting activity was inhibited only to the extent of 22%. DFP also inhibited proteolytic activity completely, whereas the above chemical agent had very little effect on milk clotting activity.

1.5. *Serratia marcescens*

Gorini (1932) and Wahlin (1933) showed that the milk clotting enzyme obtained from *S. marcescens* was thermostable and temperature, optimum for milk clotting activity was at 40-50°C. Both calf rennin and the *S. marcescens* enzyme were influenced in a similar manner by calcium ions. Oxalate was shown to inhibit the milk clotting activity to a lesser extent than in case of calf rennet. *Serratia marcescens* enzyme coagulated heat treated milk more readily than did animal rennet (Veringa, 1961).

1.6. *Streptococcus faecalis* var. *liquefaciens*

Somiuti and Babel (1965) reported that the extracellular proteinase produced by *S. faecalis* var. *liquefaciens* had optimum activity at pH 7.4 and 35°C. The proteolytic activity which
was much greater than that of rennin, chymotrypsin, trypsin or pepsin was almost completely destroyed by heating the enzyme at 85°C for 5 minutes.

2. Mould milk clotting enzymes

A large number of moulds have been reported to produce milk clotting enzymes which are mostly acid proteases. The properties of individual mould enzymes and the applicability of these in cheese industry as rennet substitutes have also been studied extensively by several workers. A brief account of the salient findings is presented in this review.

Endothia parasitica enzyme is an acid protease, which is destroyed within 5 minutes at 60°C and it is stable in a pH range between 4.0 to 5.3, with an optimum at 4.5. The isoelectric point for the above enzyme is at pH 5.5 (Sardinas, 1968), while Hagemeier et al. (1968) reported an isoelectric point of less than pH 4.6. The molecular weight of the enzyme has been estimated to be in the range of 34,000 to 30,000. The pH optima of the purified enzyme on acid-denatured hemoglobin and casein is 2.0 and 2.5, respectively. Over a pH range of 5.1 to 6.5, the milk clotting activity was much less sensitive to the pH of the substrate than that noticed with animal rennet (Larson and Whitaker, 1970a). The maximum stability of the enzyme was at pH 3.8 to 4.5. At 50°C for 30 minutes, only 30% of the activity of the enzyme was lost (Larson and Whitaker, 1970b).
Whitaker (1970) found that the enzyme is composed of the following amino acids: ala, arg, asp, asp3, 1/2lys, glu, gly, his, ile, leu, leu, lys, met, phe, ser, thr, trp, tyr, val. The above result is at variance with the findings of Sardinals (1968) who did not find any lysine, methionine or tryptophan in the crystalline preparation of the enzyme studied by him. *Endothia parasitica* enzyme is denatured by urea but mercaptoethanol, iodoacetamide, N-ethylmaleimide, p-chloromercuribenzoate, cysteine and the chloromethyl ketones derived from *N*-tosyl-L-phenylalanine and *N*-tosyl-L-lysine did not affect enzyme activity (Whitaker, 1970). *Endothia parasitica* enzyme differed from animal rennet in its preferential hydrolytic action on casein and it hydrolyzed different fractions of casein in the following order of preference: S-casein, B-casein and K-casein. The K_m for hydrolysis of K-casein by *E. parasitica* enzyme was 100 times lower than that obtained with animal rennet. The ratio of milk clotting activity to proteolytic activity of the *E. parasitica* enzyme was higher than that of other microbial rennets (Sardinals, 1972).

Ottensen and Rickert (1970a,b) characterized the enzyme produced by *Mucor miehei* as an acid protease with an optimum activity at pH 4.5 on denatured hemoglobin and at pH 4.0 on the B-chain of oxidized insulin. The isoelectric point of *Mucor miehei* enzyme is at pH 4.2, while the molecular weight is 32,000. The amino acid composition of *M. miehei* enzyme (calculated on the basis of one residue of histidine) is as follows:
ala\textsubscript{15}, asp\textsubscript{23}, l/\textsubscript{3}lys\textsubscript{2}, glu\textsubscript{14}, gly\textsubscript{19}, his\textsubscript{1}, ileu\textsubscript{11}, leu\textsubscript{12}, lys\textsubscript{5}, met\textsubscript{4}, phe\textsubscript{13}, pro\textsubscript{10}, ser\textsubscript{21}, thr\textsubscript{17}, try\textsubscript{2}, tyr\textsubscript{10} and val\textsubscript{15}. Sternberg (1971) reported that \textit{H. michei} enzyme was not metal dependent nor does it possess serine or SH active groups. The above enzyme was stable over the wide range of pH from 2.0 to 6.0 at a temperature of 40°C for 24 hr.

Calcium ions protected the activity of the enzyme to some extent. Charles \textit{et al.} (1970) claimed that the ratio of milk clotting activity to proteolytic activity exhibited by \textit{H. michei} NRRL 3432 was highest among microbial rennets.

Detailed information on the properties of \textit{Husor pusillus} coagulant is provided by an excellent review by Arima \textit{et al.} (1970). Huang (1970) also studied the enzyme in greater detail. The above enzyme is an acid protease possessing a somewhat greater thermostability than animal rennet. The crystalline \textit{H. pusillus} enzyme optimally digested β-casein at pH 4.5 and hemoglobin at pH 4.0. The isoelectric point of this enzyme was in the neighbourhood of pH 3.5 to 3.8. The enzyme was stable in the range of pH 4.0 to 6.0, peaking at pH 5.0. The molecular weight of \textit{H. pusillus} enzyme has been estimated as 20,000 to 30,000. The amino acid composition of the enzyme is as follows: ala\textsubscript{15-17}, asp\textsubscript{23}, l/\textsubscript{3}lys\textsubscript{2}, glu\textsubscript{14}, gly\textsubscript{19}, his\textsubscript{1}, ileu\textsubscript{11}, leu\textsubscript{12}, lys\textsubscript{11-13}, met\textsubscript{3}, phe\textsubscript{13}, pro\textsubscript{14}, ser\textsubscript{21}, thr\textsubscript{17}, try\textsubscript{2}, tyr\textsubscript{10} and val\textsubscript{24}.

Some metal ions inhibited the activity of \textit{H. pusillus} enzyme (e.g. ferric, mercuric, zinc etc.). Milk clotting activity of the above enzyme is increased appreciably.
by calcium ion concentration than animal rennets. Yu et al. (1971) have related histidine residues in \textit{M. pusillus} enzyme to the active site. Trop and Pinsky (1971) reported that addition of \textit{M. pusillus} rennet to animal rennet caused an increase in milk clotting activity to a greater extent than could be expected on the basis of the sum of their separate activities. This synergistic effect suggested that each enzyme presumably coagulated milk by different mechanisms.

III. USE OF MICROBIAL RENNET SUBSTITUTES IN CHEESE MANUFACTURE

A number of reviews and popular articles have been published in recent years on the use of rennet substitutes of vegetable and microbial origin in cheese making (Greenberg, 1955; Dewane, 1960; Veringa, 1961; Babbar et al., 1965; Mann, 1967; Naudts, 1969; Sardinas, 1969, 1972; Christensen, 1973; Nelson, 1973; Martens and Naudts, 1973, 1975; Green, 1977; Phelan, 1977). Such studies have been conducted in the laboratory as well as commercially using the milk clotting enzymes obtained from \textit{Nucor pusillus} Lindt., \textit{Nucor mishei}, \textit{Endothia parasitica}, \textit{Bacillus cereus}, \textit{Bacillus subtilis}, \textit{Bacillus polymyxa} and \textit{Bacillus mesentericus}. The suitability and acceptability of the microbial rennet substitutes were also assessed in relation to calf rennet.

1. Preparation of cheese using bacterial rennet

The milk coagulant of \textit{Bacillus subtilis} is one of the enzyme most extensively studied among bacterial rennets. Since
Shimwell and Evans were first granted a patent in 1944 for the production of this enzyme for cheese making, subsequently, other patents have also been issued (Murray and Kendall, 1969; Murray and Prince, 1970) as a result of interest in the enzyme shown by John Labatt Ltd., of Ontario, Canada. Using *B. subtilis* rennet, production of good quality Cheddar cheese free from bitterness has been claimed by these patentees and some other workers (Singh et al., 1967; Srinivasan et al., 1962a, b).

Emanuilloff (1956) converted about 500 liters of ewe's milk into Kashkaval and Brinza cheeses of satisfactory quality using bacterial protease. The above authors recommended that this enzyme could be used in cheese industry as a calf rennet substitute. Mauds (1969) has also briefly reviewed some of the favourable cheese trials conducted with rennet from *B. subtilis*. In an earlier review, however, Devane (1960) noted that the protease produced from *B. subtilis* gave a bitter cheese due to the accumulation of peptides during the ripening process.

The milk clotting enzymes produced by *B. cereus* and *B. subtilis* have also been studied by Srinivasan et al. (1962a) for their use in Cheddar cheese preparation. Cheese examined after 4-5 months of ripening was found to be free from bitter taste, although samples were somewhat hard and had acid taste. Good Cheddar cheese was also prepared with *B. subtilis* FR-2 rennet (Srinivasan et al., 1962b). The cheese was not only free from bitter taste but was similar in most respects to
those prepared from calf rennet. Fat loss in the whey was more in the case of bacterial rennet cheese as compared to calf rennet cheese. Chakravorty et al. (1966) prepared Cheddar cheese using bacterial enzymes from 4 isolates belonging to the *B. subtilis* group and compared these cheeses with animal rennet cheese. The degree of proteolysis was generally greater when the bacterial enzyme was used. Organoleptic evaluation did not show any marked differences between the two types of cheeses, although the flavour of cheese made from rennet obtained from one isolate appeared to be bitter.

Singh et al. (1967) prepared Cheddar cheese using enzymes derived from selected strains of *B. subtilis*, *B. megaterium* and *B. cereus*. Organoleptic evaluation revealed no significant differences in experimental and in control calf rennet cheese blocks. The flavour was found to be acceptable, and no bitter taste was noticed at any stage of ripening. Analysis of the whey samples showed a total solid content which was slightly higher than in control calf rennet cheese samples. In laboratory trials, Schalinatus and Behnke (1967) found a higher proteolytic titration and fat content in whey obtained after treatment of milk with different rennets produced by *B. subtilis* and *B. cereus*. Cheddar cheese made with protease complex obtained from a mutated strain of *B. subtilis* was of excellent quality, but the sweet types of cheese showed a higher degree of proteolysis and off-flavour development. Yields tended to be lower because of the high proteolytic activity of the enzyme (Irvine et al., 1969). Murray and Kendall (1966) obtained a
patent for their process on the production of cheese with excellent texture, flavour stability characteristics and high yields using an enzyme complex produced by _B. subtilis_.

Nasek _et al._ (1970) concluded that cheeses made from rennet preparations of 3 strains of _B. subtilis_ were not as good as those made from calf rennet, particularly in regard to coagulum firmness. The above workers recommended use of larger quantities of these enzyme preparations in admixture with calf rennet and also suggested heating of the coagulum for increased firmness of the curd.

Murray and Gruetzner (1970) recommended some technological changes during the manufacture of Cheddar cheese using an enzyme preparation derived from _B. subtilis_. For maintaining moisture and firmness within the limits normally obtained with rennet, they suggested application of heat during the first 30 minutes of the 45 minutes holding time, and also recommended the raising of the temperature of curd to 110°C for 3 minutes, followed by quick cooling to the normal clotting temperature of 103-104°F.

John Labatt Ltd. (1971) have described a method of Cheddar cheese manufacture involving the use of a complex of neutral and acid peptidase derived from _B. subtilis_ for coagulating milk which had been pre-ripened with a commercial starter for 30 minutes. The resultant cheese with a moisture content of 35% was found to have an excellent texture. Organoleptic and chemical examinations during 180 days of ripening and storage of model cheeses (Annental type) made with protease of _Bacillus subtilis_ showed that these were
unsuitable for the production of hard cheese (Puhan and Steffen, 1967). Puhan and Irvine (1973a) reported, during the course of their studies on the manufacture of Canadian Cheddar cheese, that proteolysis by bacterial proteases singly or together was continuous resulting in a softer curd with more protein losses in whey than that observed in the control batches of calf rennet cheese. The fat losses in whey were also relatively higher in the experimental lots. More proteolysis by bacterial proteases lowered the yield of cheese by 10%. It was also found that the cheese made with proteases from a mutant of \textit{B. subtilis} revealed less proteolytic activity on milk proteins when pH of the milk was lowered by the addition of acid (Puhan and Irvine, 1973b). Doležalek \textit{et al.} (1974) found that the quality of \textit{Zlato} cheese prepared by using \textit{B. subtilis} preparation ("Mikrozym") was considerably lower than the quality of cheese produced with calf rennet ("Laktocynam").

The rennet prepared from \textit{Bacillus polymyxa} has also been used for cheese manufacture but with limited success. The quality of the cheese made from bacterial rennet was reported by Godo Shusei Co. (1969) and Imai \textit{et al.} (1972) to be similar to that produced using commercial animal rennet and had no bitterness up to 4 months of ripening, whereas Phelan (1973) assessed the cheese from bacterial rennet to be unacceptable quality. "Milcozym", a commercial rennet substitute from \textit{B. polymyxa} has also been considered unsuitable
for the production of Cheddar, Tilsit and Kortowski cheeses by Reps et al. (1975) due to excessive proteolysis in the curd, thereby resulting in low quality product.

Barkan (1964) reported that cheese made with the milk clotting enzyme obtained from Bacillus mesentericus showed a relatively rapid protein breakdown. The soluble and amino nitrogen after 3 months of ripening was 50-100% higher than in control animal rennet cheese. Organoleptically, B. mesentericus rennet cheese had a typical flavour resembling that of Brinza cheese, but it often had a bitter taste, and was harder and more crumbly than control calf rennet cheese.

The results of cheese making trials by Antila and Aspola (1970) using the bacterial proteases were generally satisfactory, although the bacterial proteases cheese blocks often exhibited specific flavour or body characteristics. Kachkaval and White cheeses prepared with the bacterial enzyme and preserved in brine solution were similar to the control batches of calf rennet cheese (Stefanova-Kondratenko and Andonova, 1972). Dimitroff and prodanski (1973) manufactured White cheese and preserved it in salt brine using B. mesentericus preparation and also calf rennet. While bacterial rennet cheese was found unsuitable when made with cow's milk, no such differences were observed during production or ripening of cheese made with sheep's milk. In case of Kachkaval cheese, there was no quality differences after prolonged ripening.
in spite of initial mealy structure noted in the cheese. Mikhailus and Popova (1975) prepared "Bryndza" cheese on a pilot scale by comparing the enzymes "Misanterin" or B. *mesentericus* rennet with standard calf rennet. No quality differences were found in cheeses thus manufactured by using the above enzymes. In some cases, however, the experimental batches of cheese exhibited a softer consistency. Parallel tests carried out on an industrial scale using B. *mesentericus* enzyme showed that it was perfectly suitable for production of Bryndza cheese (Mikhailus et al., 1975).

Hard cheeses were made using the milk clotting enzyme preparations from some selected strains of B. *subtilis* and B. *mesentericus*. Satisfactory cheeses could be obtained from strains 4; 4,232; 69 and 15 (Antonova et al., 1975).

Srinivasan et al. (1968) observed that the Cheddar cheese prepared from milk clotting enzyme of *Streptococcus liquefaciens* was of good quality and showed no bitter taste after one month of ripening.

2. Preparation of cheese using mould rennet

Many types of cheeses have been successfully prepared using *Endothia parasitica* rennet. In fact, some cheeses which have been produced by using the above enzyme are superior to those prepared with the animal rennet. The types of cheeses prepared with satisfactory quality using *E. parasitica* rennet
include Brie, Camembert, Cheddar, Colby, Emmentaler, Gruyere, Italian varieties, Limburger, Monterey, Munster, Swiss, etc. (Bolliger and Schilt, 1969; Carini and Todesco, 1974; Puhan and Steffen, 1967; Ramet and Schluter, 1970; Ramet et al., 1969; Shovers and Bavisotto, 1967). In contrast to the above findings, Phelan (1973), Reps et al. (1975) and Carbone et al. (1976) showed that Taleggio cheeses produced by using E. parasitica enzyme, were of inferior quality and had bitter taste.

Kikuchi et al. (1968b) and Resmini et al. (1971) have suggested some minor modifications in cheese making technique using Endothia enzyme.

Maubois and Hocquot (1969) observed a one percent loss in yield by using E. parasitica rennet for production of Camembert type cheese. On the other hand, Ramet et al. (1969) obtained slightly higher yields of Camembert cheese using the above enzyme.

Several workers have reported preparation of variety of cheeses using K. miehei rennet. These cheeses include, Cheddar, Domati, Emmental, Italian (soft), Jarlsberg, Kachiaval, Limburger, St. Paulin, Tollenser, Trappist and White (Arnes, 1971; Behnke and Siewert, 1969; Carini and Todesco, 1974; Christensen, 1972; Dinesen et al., 1975; Hamdy, 1970; Mayer, 1971; Phelan, 1973; Prins and Nielsen, 1970; Ramet and Alais, 1973; Sipka et al., 1974; Wigley, 1974).
Prins (1973) has recommended reduction in the quality of enzyme and a slight increase in calcium chloride content in milk for the manufacture of better quality cheeses made from H. michei rennet. It has been claimed that good quality cheese can be produced with Rennilase at 45% lesser cost (Thompson et al., 1972). Use of Rennilase has also been recommended in cases where starter cultures along with animal rennet are known to be responsible for bitterness in Cheddar cheese (Lawrence et al., 1972).

Several defects such as bitterness, slight delay in the hardening of the curd, doubtful flavour, mealy structure in cheese manufactured by using H. michei rennet have also been reported (Aapola et al., 1973; Antila and Aapola, 1975; Behneke and Siewert, 1966; Dolezalek et al., 1974).

There have been satisfactory reports in regard to preparation of cheese using H. musillus rennet. Several workers have prepared cheeses such as Brazilian, Brick, Butter, Camembert, Cheddar, Cottage, Edam, Gouda, Grana, Italian, St. Paulin, Taleggio, Tilsit and Yugoslavian using H. musillus enzyme (Alberini and Nizzola, 1975; Annibaldi and Nizzola, 1969; Arima, 1972; Arima et al., 1970; Carbone, 1976b; Kyle-Surola and Antila, 1970; Phelan, 1973; Richardson et al., 1967; Robertson and Gilles, 1969; Sandoval et al., 1972; Schulz et al., 1967; Wigley, 1974; Zwaginga et al., 1969).

Kikuchi et al. (1968), Pederson (1969), Tsugo et al. (1964) and Yu et al. (1971) have reported production of
acceptable cheese by slightly modifying cheese making procedure by the addition of calcium chloride prior to renneting or by using the crystalline enzyme.

There are some other reports which indicate bitterness, body defects, low yields and higher fat losses in whey during cheese manufacture using *M. musculus* rennet (Antila and Aapola, 1969a, b; Aapola et al., 1973; Babel and Somkuti, 1968; Carbone et al., 1976; Kimuchi and Toyoda, 1969; Kimuchi et al., 1968a, b; Repa et al., 1975).

3. Preparation of cheese using combination of microbial rennet with other enzymes

The research committee of the National Cheese Industry (U.S.A.) in 1960 have advised that traditional rennet should be replaced only partially by pepsin and that this procedure should be limited to cheese that has to be stored for long periods (Veriga, 1961). Since commercial rennet contains both rennin and pepsin, it is likely that pepsin may prove a suitable rennet substitute. Studies on cheese making by Maragoudakis et al. (1961) and Melachouris and Tuckey (1964) have indicated that swine pepsin can be used as a rennet substitute provided that the cheese is matured for a longer period than what is necessary with calf rennet. Raadsveld (1964) observed that cheese made with pepsin ripened somewhat slowly so far as proteolysis was concerned.

According to Babel (1967) about 75% Cheddar cheese produced in U.S.A. is made by using a mixture of pepsin and
rennin which gives the cheese an excellent quality. Chapman and Burnet (1968) have also prepared Cheddar cheese in
different laboratory trials for nearly a year, using a mixture
of pepsin and animal rennet. Bovine pepsin has been shown
as a more suitable rennet substitute for calf rennet (Fox, 1969).

Nelson (1969) prepared Cheddar, Swiss, Pasta, Filata,
Hard grating and mould ripened cheeses with fungal enzyme
and compared them with cheeses made with calf rennet and
calf rennet-pepsin mixture (50:50). All the enzyme preparations
gave cheeses of acceptable quality with no differences in
yield. Protein breakdown and flavour development with the
fungal enzyme was faster than with animal rennet. Cheese made
with rennet-pepsin mixture showed the slowest ripening.

Hartens and Naudts (1973, 1976) and Green (1977) have
also suggested use of 50:50 mixture of rennin and swine pepsin
for manufacture of different types of cheese, since no
significant differences were reported between cheeses made with
calf rennet or with rennet-pepsin mixtures.

Masek et al. (1974) compared 'Hikrozym' cheese with
calf rennet cheese from five dairies and found that a 1:1 or
1:1 mixture of 'Hikrozym' and calf rennet gave good results.

Cartone et al. (1976a) carried out several tests with
Taleggio and Italiano cheese, using Rennilase and a standard
rennet mixture in separate trials. The former did not show
any unfavourable influence on the organoleptic quality of cheese but reduced the yield. However, Rosmini et al. (1976) stated that Italico cheese manufactured with Promase had a more pronounced flavour and a slightly softer consistency than control cheese obtained with calf rennet. When a mixture of calf rennet-Promase was used as the coagulant, the above defect in cheese was significantly reduced.

Morris and McKenzie (1970) produced good Cheddar cheese using a 1:1 mixture of Endothia and calf rennet. But cheese quality was reduced when the ratio of the two enzymes was 25:75. Bitterness was observed in Edam, Tilsit and Butter type of cheeses when more than 70% Endothia rennet was substituted for calf rennet (Thomasow et al., 1970).

The assessment of curd quality, flavour and consistency of Cheddar cheese favoured a mixture of Soury rennet from M. pusillus Lindt. and swine pepsin in 30: 70 ratio (Green and Stockpoole, 1975). The above workers also studied the behaviour of several Mucor pusillus Lindt. rennet preparations and swine pepsin mixtures and compared them with calf rennet for Cheddar cheese production. Cheese prepared with an admixture of swine pepsin and Mucor pusillus rennet was of higher quality and was comparable to calf rennet cheese than when preparation of cheese was based entirely on the use of M. pusillus rennet.
Resmini et al. (1976) noted that *M. pusillus* Lindt, rennet-based Italico cheeses showed a more pronounced flavour and a softer consistency than calf rennet cheese. The defect was, however, decreased by using a mixture of *M. pusillus* rennet and calf rennet in 50: 50 ratio.