DISCUSSION

Microorganisms, particularly molds, are used extensively for industrial production of several enzymes like amylase, dextrinase and protease. Recently, attempts have been made to exploit several species of bacteria and molds for the production of milk clotting enzymes which can partially or completely replace the animal rennet. This has become necessary in view of the high cost as well as world shortage of animal rennet required for cheese manufacture. The present study has indicated the possibility of using a mold culture, isolated locally, for the production of milk clotting enzyme with low proteolytic activity.

I. Isolation and selection of Molds for production of Milk Clotting Enzyme

Ninety mold strains have been isolated from various sources and screened for the production of milk clotting enzymes. Out of these only twenty-nine were capable of producing this enzyme (Tables 1 and 2).

An ideal rennet preparation should not only possess good curdling capacity, but also have a high ratio of milk clotting to proteolytic activity. Among the 29 isolates which showed milk
clotting activity, one mold strain obtained from wheat bran showed a ratio of high milk clotting to proteolytic activity besides having considerable milk clotting ability. The strain was placed in the species of *Absidea rennosa* on the basis of its growth and morphological characteristics (Smith, 1960) and it was selected for further studies. Such low incidence of strains having satisfactory properties for use in rennet production indicates the necessity for a wider screening of microorganisms to get suitable strains with maximum economic feasibilities. Similar investigations on a larger group of microorganisms have been carried out by Arima *et al.* (1967) who selected one strain of mold, *Rhizopus niveus Lindt* after screening more than 827 isolates, including molds, actinomycetes and bacteria. Many investigators in different parts of the world have screened a large number of microorganisms for this purpose (Knight, 1966; Sardinas, 1967; Kawai and Mukaé, 1970; Su and Chen, 1970; Yu *et al.*, 1971; Abdel-Fattah *et al.*, 1972; Kawai, 1973) and the search is still in progress for selection of suitable organisms from a variety of other sources.

In India, Srinivasan *et al.* (1964) and Babber *et al.* (1965) have conducted extensive survey on isolation and screening of bacteria and molds for this purpose. But as they obtained certain strains of bacteria capable of producing potent rennet substitutes much emphasis was not put on the selection or development of mold strains for this purpose.

### II. Factors Influencing the Production of Milk Clotting Enzyme by *A. rennosa*

It is well known that the growth and biosynthetic activities
of microorganisms vary considerably according to availability of the required nutrients, presence of various activating or inhibitory substances or environmental conditions. It is therefore, essential that when a strain is to be selected for use its performance under different conditions has to be tested and the optimum requirement for maximum yield of the product determined. With this end in view, the study of certain environmental conditions and nutritional factors have been conducted with the selected strain \( A. \) \( remora \).

(i) Environmental Factors

(a) Shake culture cultivation: Several media were tried in different combinations for the purpose of production of milk clotting enzyme by \( A. \) \( remora \) (Table 3). Except for 5 per cent wheat bran medium containing minerals from Czapec medium with or without yeast extract, the milk clotting enzyme was not produced in the media tried. This shows that although dextrose and corn steep liquor support the growth of the organism, they lack the essential nutrient in the form of salts or accessory growth factors. The growth and enzyme production in wheat bran medium also indicates the possibility of presence of a precursor which is essential for synthesizing the milk clotting enzyme. The production of enzyme was however lesser in these liquid media than on semisolid medium.

Semisolid wheat bran has been the medium of choice of many workers for the production of enzymes from molds since early days (Takamine, 1894, 1914 and Underkofler, 1947). Even in recent years Yipogof and Patiia (1966), Arina et al (1967), Yu et al (1971) have found semisolid media containing wheat bran suitable for
enzyme production by *Asp. candidus*, *Mucor pusillus*, *Ochroclarella rubra*, respectively.

Liquid media have also been extensively used for the production of mold enzymes, either under static or shaking conditions with and without aeration. The yield of enzymes have varied widely according to the conditions of cultivation. Maxwell (1982) reported maximum yield of protease from a still culture of *A. flavus* enzyme with a volume to surface area ratio of 0.9. A strain of *M. pusillus* was grown in a liquid medium consisting of whey or 4% wheat bran by Babal Reddy (1968). Rafa and Pazněk (1970) obtained maximum yield of milk clotting enzyme from *Byssochlamys fulva* in a static liquid medium with a pH of 5 – 7 after 10 to 12 days at 25 – 30°C, while Chu et al (1973) obtained the same activity in 4 days by shaker cultivation.

Sardina (1967) obtained maximum yield of milk clotting enzyme from *Endothia parasitica* by pot fermentation with an aeration rate of 0.5 vol/vol/min and an agitation rate of 1750 rev/min for 48 hours. The medium consisted of soya bean meal, aerolose, matrix skim milk, sodium nitrate, potassium dihydrogen phosphate and magnesium sulphate with a pH of 6.0.

*Mucor miehei* ATCC 16457 has also been shown to produce large quantities of milk clotting enzyme in a liquid medium by Baxter Lab Inc (1971).

Gripon and Bergere (1972) produced proteolytic enzymes from *Penicillium requartii* and *P. frequentans* in 3 – 5 days at pH 6.0
on tryptase, glucose, minerals medium by shaking. Abdel-Fattah
\textit{et al.} (1972) observed higher milk clotting enzyme production in
still cultures of \textit{P. uncinatum} grown on 2% maize steep liquor and
2% lactose at 30°C. The production of proteolytic enzyme was
higher than clotting enzyme in shaker cultivation.

These results indicate the existence of strain variations in
the production of the enzyme as well as variations in their growth
requirements. The failure of \textit{Abaedie ramona} to give higher yield
of milk clotting enzyme in liquid media compared to semisolid media
may be due to lack of certain nutrients in the media tried in the
optimum concentration or to its inherent characteristics. Further
investigations are however needed to prove this hypothesis.

In view of the higher yields of enzyme obtained with the semi-
solid wheat bran medium, the influence of different environmental
factors was studied employing the same medium for cultivating the
mold strain.

(b) **Temperature and period of incubation**: \textit{Abaedie ramona} gave
maximum yield of milk clotting enzyme when it was grown at 22°C
for a period of 96 hours (Table 4 and Fig. 1). It was, however,
observed that the optimum temperature for the growth of the organism
was 37°C.

The temperature and period of incubation required for maximum
enzyme production in the case of other organisms reported by earlier
workers vary according to species of organism used and their
general growth requirements (Tipografia and Patina, 1966; Arima \textit{et al.},
The observations made regarding the higher yield of enzyme when the culture was initially incubated at 30°C for 24 hours followed by subsequent incubation at 22°C was probably due to the increased initial growth and reduction in lag phase at the higher temperature (Table 5). The above observations agree with the findings of Chipizubova and Grechava (1968) who noted higher yield of protease and amylase in Asp. oryzae by changing the incubation temperatures at different stages of growth. Nakanishi and Itoh (1971) adapted this technique for higher yield of protease in Asp. oryzae chosen to reduce the incubation period from 8 days to a total of 87 hours.

On continuous incubation at 30°C or 37°C, for a further period of 24 hours A. oryzae showed a reduction in the yield of enzyme (Table 4 and 5, Fig. 1). This may be due to the action of other proteolytic enzymes produced by the organism on these proteins bringing about their inactivation. This technique of changing the incubation temperature at different stages of growth may prove economical in terms of both time and cost.

(c) Moisture content: The moisture content of the wheat bran medium had profound influence on the production of milk clotting enzyme by A. oryzae, the optimum level being fifty per cent without forced aeration. When this was increased to 75 per cent the enzyme yield decreased by over 60 per cent (Table 6, Fig. 2).

The moisture requirement of other molds for enzyme production have been reported to vary from 30 to 80% while Pithierella ribis required 60 - 80% (Yu et al., 1971). Ruscop novillius required only
35 to 45% (Arina et al., 1967). Aspergillus candidus gave maximum yield of enzyme at 58 – 60% moisture content with forced aeration but needed only 50 – 55% when forced aeration was not provided.

The optimum moisture requirement of A. reqes for enzyme production was within the range prescribed by Kalashnikov et al. (1966) for mold cultures which was between 50 and 60 per cent.

(c) pH: Hydrogen ion concentration was another factor which had pronounced influence on the growth and enzyme production by this mold. Although pH 4.0, 6.0 and 7.0 were relatively more suitable for enzyme production, the organism was capable of producing considerable amount of enzyme over a wider pH range of 3 – 8. For protease production media with 6.0 and 7.0 pH were better for this mold (Table 7 and 8).

R. pusillus produced maximum acid protease in a liquid medium adjusted to pH 5.0 (Sakut and Babel, 1967) while when cultivated on semisolid wheat bran medium it produced the milk clotting enzyme at a pH range of 5 – 7 (Arina and Iwasaki, 1969). A. candidus required a pH of 6.0 (Dimitrov et al., 1969) for milk clotting enzyme production while L. parrsitus produced maximum enzyme at pH 4.7 (Rape and Poznaki, 1970). Highest yield of enzyme was obtained by Hashimoto et al. (1973) in R. duponti using a pH range of 4.0 to 5.5 in the medium.

(ii) Nutrients

(a) Carbohydrates: Among the supplements tested, sugar
sources like dextrose (2.0%) or molasses (2.0%) favoured increased enzyme production by 50% and 45.5% respectively (Tables 9 and 11 and Fig. 3) by A. ramannii, while supplementation of starchy sources (0.25% starch) resulted in appreciable fall in the yield of milk clotting and proteolytic enzymes (Table 10 and Fig. 3).

It may be mentioned here that A. ramannii was capable of producing appreciable quantities of amylase (Table 8 and Fig. 12) and the reduction in the production of proteases in the presence of starch may be due to substrate induced amylase production.

Similar increases in yield of milk clotting enzymes by Dethiobacter ribis and P. citrinum have been obtained when the medium contained 3% sucrose and 2% lactose respectively (Yu et al., 1974; Abdel-Fattah et al., 1972). Shigeji et al. (1957) found an increase in acid protease production by Aeg. oxytes in wheat bran medium and amylase in rice medium. They further proved that by addition of a protein as an inducer in the rice medium the organism can also be made to produce proteases. Rice added with milk was used by Kahman et al. as early as 1927 for the production of milk clotting enzyme.

(b) CHLORIDE AND CITRATE: Sodium chloride or calcium chloride in 0.2 per cent concentration in the medium were found to increase the yield of the milk clotting enzyme by A. ramannii. The increase was by about 26% with sodium chloride and by 5% with calcium chloride. When the medium supplemented with magnesium citrate (0.1%) was used for cultivation the yield of milk clotting enzyme decreased by 46 per cent (Tables 12 and 13 and Fig. 4).
The results obtained in the case of A. ramosa agree with the report of Samkuti and Sabel (1969) who got highest release of acid protease from Mucor pusillus by incorporation of 0.5 M NaCl in 4% wheat bran medium. Yu et al. (1971) used a medium with sodium chloride and potassium dihydrogen phosphate as inorganic salt supplements for the production of milk clotting enzyme by Pediococcus ribis.

Magnesium salts are included in many media used for enzyme synthesis by molds but in the case of A. ramosa these salts were inhibitory to the production of milk clotting enzyme. The slight increase of milk clotting activity by calcium chloride may be due to the influence of Ca ion present in the extract on the milk clotting activity of the enzyme during testing.

(c) Nitrogen sources: Nitrogen compounds in inorganic or organic forms are known to be involved in the growth and enzyme synthesis by microorganisms.

Among the inorganic sources of nitrogen tried, addition of ammonium sulphate to wheat bran medium resulted in an increase in the milk clotting enzyme production by A. ramosa by 21 per cent whereas incorporation of sodium nitrate resulted in a decrease by about 47 per cent. There was however no effect on the yield of protease. With ammonium nitrate supplementation about 11 per cent in the yield of milk clotting enzyme was observed but yield of protease increased by 21 per cent (Table 14, Fig. 5).
Sodium nitrate has been used in liquid media without any adverse effect by Sardinas (1967) and Reza and Poznanski (1970) for large scale production of milk clotting enzyme by \( A. \) _parasitica_.

In the present study, it may be noted from earlier results (Table 3) that supplementation of salts of Czapek medium (which contains 2% sodium nitrate) to liquid wheat bran medium facilitated good growth and also a little enzyme production by \( A. \) _parasitica_ but incorporation of sodium nitrate individually even at 1% level to semisolid wheat bran medium showed an adverse effect on the enzyme production. Plain salt solution of Czapek medium also did not support growth and enzyme production under shaking conditions of cultivation. Since the environmental conditions of cultivation in these experiments are different, the results are not comparable for drawing definite inferences. However, from the above studies it is obvious that \( A. \) _parasitica_ does not release milk clotting enzyme in the same extent in the presence of nitrate ions as with sulphate ions and nitrate is inhibitory to its growth and production.

All the predigested protein supplements tried increased the yield of milk clotting and proteolytic enzymes of \( A. \) _parasitica_. The increase in milk clotting enzyme production was fifty per cent with peptone or protease peptone. The increase was relatively lesser with tryptone (Table 15, Fig. 5).

These results indicate that some of the lower chain peptides or amino acids present in protease peptone or peptone (but probably absent in tryptone) stimulate the enzyme production by \( A. \) _parasitica_. Similar observations have been made by Konevalov _et al_ (1967) who
obtained a higher milk clotting enzyme production in \textit{Asep. oryzae} or \textit{Asep. flavus} by replacement of sodium nitrate in Czapek medium with casein or peptone. They noticed stimulation of enzyme synthesis by aspartic acid and lysine and inhibition by tyrosine, methionine and arginine. Some of the amino acids although increased growth were inhibitory to enzyme production. Derakhov and Konevlev (1969) noticed a definite correlation between the intracellular free amino acid, particularly glutamic acid and acid protease synthesis in the above molds.

Srinivasan \textit{et al.} (1968) used papain digested skim milk for higher yield of milk clotting enzyme by \textit{A. oryzae} and \textit{A. subtilis}. Increase in the enzyme production by \textit{Str. liowafangian} was noticed with the addition of protein, peptone or casein amino acids by Srinivasan \textit{et al.} (1968).

\textbf{(d) Corn Steep Liquor:} Addition of corn steep liquor to wheat bran semi-solid medium resulted in a decrease in the production of milk clotting enzyme by \textit{A. oryzae} (Table 16 and Fig. 6). Corn steep liquor medium has, however, been used for enzyme production by other molds e.g. \textit{Pan. citrinum} (Abdel-Fattah \textit{et al.}, 1972).

\textbf{(e) Skim milk:} Plain skim milk or skim milk in a digested form when incorporated into liquid medium has been reported to stimulate the production of milk clotting enzyme by many organisms (Kahsen \textit{et al.}, 1927; Shigaji \textit{et al.}, 1957; Srinivasan \textit{et al.}, 1962, I Sardinas, 1967; Farashungains, für die Garugsind, 1970).

In \textit{Abedula remos} also an increase in the production of milk
clotting enzyme was observed by the addition of skim milk to the semisolid wheat bran medium up to a level of 25%. There was more than 45 per cent increase in the concentration of milk clotting enzyme and 3.4 per cent increase in the case of proteolytic enzyme (Table 17 and Fig. 6).

(f) Malt Extract: With the increasing concentration of malt extract in wheat bran medium there was an increase in the production of milk clotting and proteolytic enzymes by A. FANOSA. While the increase in milk clotting enzyme was 41.6 per cent, the increase in proteolytic activity was only by 17.4 per cent (Table 18 and Fig. 6).

(g) Minerals: Supplementation of wheat bran medium with the minerals of Czapek Dox medium showed a decrease in the yield of silk clotting enzyme by A. FANOSA (Table 19). As already pointed out this mineral mixture consists of sodium nitrate and magnesium sulphate which were shown to inhibit the milk clotting enzyme production (Table 13 and 14 and Fig. 5).

(iv) Inoculum

Growth medium used for preparation of inoculum showed an influence on the production of milk clotting enzyme by A. FANOSA (Table 19). When transferred from wheat bran medium to wheat bran medium the organism did not show any increase in the yield but when transferred from a medium with mineral supplements to bran medium it gave higher yield. This stimulation may be due to the starvation effect produced due to lack of certain minerals in the bran medium.
Nakanishi and Itah (1971) found similar stimulation due to starvation effect in the case of *Aspergillus oryzae* chosen 8.

(v) Acid or Alkali Extraction of Wheat Bran

Use of wheat bran extracted with acid, as a medium for growth of *A. oryzae* did not affect the enzyme production whereas the bran after NaOH treatment and subsequent neutralization was not useful as a medium either for growth or for production of enzyme (Table 20). The bran when extracted with distilled water by boiling, lost some quantity of its nutrients in the extract. The extract also served as a medium for growth and enzyme production. This indicated that the nutrient responsible for enzyme production was partially soluble in water.

There has been no previous reports on the extraction of wheat bran with acids or alkali to study the growth stimulating substance in it for milk clotting enzyme production. From the above studies it was evident that the substance responsible for milk clotting enzyme production in wheat bran by this mold was alkali labile, and partially extractable by boiling in distilled water.

III. Extraction of the Milk Clotting Enzyme

Extraction of the enzyme from the culture by shaking or freezing and thawing resulted in maximum release of the enzyme (Table 21). It was observed in the present trial that agitation facilitated easy and complete extraction of enzyme from this mold.
Sonkuti and Babel (1968) separated the enzyme preparation of \( \text{M. pusillus} \) by separation of the growth through celite filtration. Sardines (1967) also obtained the enzyme by filtration as the medium used for growth was broth.

Iwasaki et al. (1967) extracted the enzyme of \( \text{M. pusillus} \) by adding 10 litres of water to 2 kg bran with the growth of the mold. The mixture was kept under 50 ml toluene for 20 hours at room temperature. Six litres of extractant was recovered.

These results indicate that the enzyme of \( \text{A. ramosa} \) is probably extracellular and may be bound to cell wall or matrix of the semi-solid medium which when shaken vigorously gets extracted.

IV. Concentration of the Enzyme

The crude extract of the growth of \( \text{A. ramosa} \) from wheat bran was concentrated without any loss in activity to 1/15th of its volume (Table 25) by vacuum evaporation within 6 hours at 40°C. This method can be used for large scale concentration of enzyme to reduce the quantity of ammonium sulphate or organic solvent needed to precipitate the milk clotting enzyme.

Similar technique has been used by Sardines (1967) to concentrate the broth filtrate of \( \text{Endothia parasitica} \). Veselov (1967) suggested a novel technique of concentrating the enzyme by freezing the enzyme solution and slightly thawing. But the recovery depended upon dry matter content of the solution and the nature of the enzyme. Rickert and Elliot (1973) used a diafiltration technique to purify
the milk clotting enzyme of *Nucor mishiei* with a recovery of 80% of its activity.

V. Purification of the Enzyme

(i) Fractional precipitation with ammonium sulphate

When the crude enzyme extract of *A. ramosa* (which was not concentrated) was precipitated with ammonium sulphate (60% w/v) about 70% milk clotting activity was recovered (Table 22). But by initially concentrating the extract to 1/15th of its volume same recovery was obtained with only 50 per cent ammonium sulphate concentration (Table 26). With this fractionation or precipitation technique nearly two fold purification of the enzyme was attained.

Iwasaki *et al.* (1967) obtained a recovery of 89.6% milk clotting activity with *B. pсеillus* extract in 30 - 50% (w/v) ammonium sulphate fraction. Somkuti and Babel (1966) used 85 per cent ammonium sulphate saturation for precipitation of milk clotting enzyme from *B. pсеillus* in the filtrate. Sardinae (1967) used only 40 per cent concentration of ammonium sulphate to precipitate the milk clotting enzyme of *E. perseptiae*. The milk clotting enzyme of *Nucor mishiei* was precipitated by treating the culture concentrate with 60% (w/v) ammonium sulphate by Ottsen and Riskert (1970). They obtained a recovery of 86% with a purity of 2.83 folds. Sternberg (1971) precipitated the enzyme from the same solid with 39.13% ammonium sulphate (w/v) by adjusting the pH to 5.2 before precipitation. He obtained a 5.01 fold purification.
The investigations carried out with *A. rana* enzyme have shown that the milk clotting enzyme of this mold precipitates between 20 to 50% w/v of ammonium sulphate concentration in 15 times concentrated extract and between 30 to 60% w/v ammonium sulphate concentration in the original extract (Tables 22 and 26).

(ii) Fractional precipitation with acetone

With the original extract of *A. rana* 1:2 volumes of acetone were needed for maximum recovery of milk clotting enzyme. The precipitation of milk clotting enzyme occurred between 0.5 and 2 volumes of acetone. With this procedure a total recovery of only 44.36% of milk clotting activity was obtained with 2.81 fold purification (Table 23). When the acetone precipitation was done after ammonium sulphate precipitation a recovery of 60% was obtained. 32% activity was lost during the initial precipitation with ammonium sulphate (Table 23). This two step precipitation also gave a purity of 6.65 folds (Table 24) and required only 1:1.5 volumes of acetone for getting complete recovery. Precipitation of the enzyme initially with ammonium sulphate probably stabilizes enzyme protein or makes it more resistant to denaturation. With the concentrated extract also, after precipitation with ammonium sulphate only 1.5 volumes of acetone was required for obtaining complete recovery of milk clotting activity (Table 27).

Hagemeyer et al (1966) also used acetone precipitation technique after ammonium sulphate fractionation for purification of milk clotting enzyme from *E. aerogenes*. Iwasaki et al (1967)
precipitated the milk clotting enzyme of _M. pusillus_ using ethanol, acetone or methanol and obtained a recovery of 76.1%, 66.8% and 78.9% respectively. These were lower than the recovery of 89.3% obtained by precipitation with 50% w/v ammonium sulphate.

(iii) Fractional precipitation with isopropanol

When the ammonium sulphate precipitate was redissolved in distilled water and reprecipitated with isopropanol, only 1.0 volume of isopropanol was necessary to give complete recovery of milk clotting activity (Table 28). Use of this solvent for purification purposes, appears to be more advantageous as the volume of organic solvent needed for precipitation is reduced.

Sardinae (1967) used the following steps for purification of the milk clotting enzyme of _L. parasitica_: Ammonium sulphate precipitation, acetone precipitation, treatment with activated carbon, freeze drying and redissolving the precipitate in distilled water, and crystallization of the enzyme with isopropanol.

(iv) Gel filtration through Sephadex G-75 column

When the enzyme of _A. remora_ was passed through a Sephadex G-75 column, the milk clotting and proteolytic activities were recovered in the first two protein peaks (Fig. 7). This showed that the milk clotting and proteolytic enzymes of _Abeidina remora_ definitely contained two fractions with two different molecular weights. Further investigation on these aspects may be helpful in revealing the nature of the proteolytic and milk clotting enzymes. With this technique
of gel filtration a purification of 10.45 fold was attained (Table 24). Gel filtration technique was useful in separating the three minor peaks of proteins without any milk clotting activity from the two major peaks containing the active fractions.

Iwaseki et al (1967) achieved a 10.7 fold purification of milk clotting enzyme from the crude extract of R. pusillus by column chromatography on Amberlite IRC-50 and DEAE Sephadex column. They found the enzyme to be homogeneous. Somkuti and Babai (1968) purified the acid protease of R. pusillus with ammonium sulphate precipitation and ethanol precipitation followed by gel filtration through Sephadex G-75. They passed this through a column of DEAE Sephadex A-50 to get a homogeneous milk clotting enzyme. Meelker and Rathijaan (1971) purified the milk clotting enzyme of R. pusillus by using silicates as adsorbents. They succeeded in separating the proteolytic fraction responsible for development of bitterness from milk clotting fraction.

Ottesen and Riekart (1970) purified the crude preparation of R. mishmi using DEAE Sephadex and S.X. Sephadex ion exchange chromatography after ammonium sulphate precipitation. This was finally passed through a column of Sephadex G-75 to get a homogeneous enzyme preparation. By these techniques they attained 14 fold purity. Sternburg (1971) recovered 37.2 per cent recovery of milk clotting activity and a homogeneous preparation of R. mishmi by ammonium sulphate precipitation, Amberlite CG-50 chromatography and CM-cellulose chromatography.
Chu et al (1973) obtained a 20 fold purification of milk clotting enzyme of *S. fulva* by ammonium sulphate fractionation, DEAE cellulose and Sephadex G-100 chromatography. They also obtained a nine fold purification of an enzyme from *Penicillium duponti* by alcohol precipitation, column chromatography on DEAE cellulose, treatment with CM-cellulose and filtration through Sephadex G-100.

Kawai (1971) partially purified an enzyme of *Irmo lactea* by 8.83 folds using CM-cellulose chromatography, ammonium sulphate precipitation and rechromatography on CM-cellulose. Hagemeyer et al (1968) obtained 5.49 fold purification of *F. parasitica* using ammonium sulphate and acetone precipitation and Sephadex G-100 and DEAE cellulose chromatography.

The foregoing references, in respect of the different methods employed for enzyme purification, point out that most of the workers have invariably adopted ion exchange chromatography for achieving a fair stage of purity of the enzyme preparations.

The results obtained in the present investigation with polyacrylamide gel electrophoresis of Absidia rennet indicate that the enzyme still has many protein fractions (Fig. 15). In order to get a homogeneous preparation of the enzyme it may be necessary here also, to use ion exchange chromatography and other absorption techniques.
VI. Properties of the Milk Clotting Enzyme

(1) Optimum pH and temperature for milk clotting activity

The enzyme of *Absidia creperi* showed maximum milk clotting activity at 55°C and pH 5.5. In respect of pH and temperature optimal *Absidia* enzyme was very similar to animal rennet. In the case of Meite rennet, although the optimum pH for milk clotting activity was 5.5, the optimum temperature was 60°C. None of the three enzymes clotted milk at pH 7.0 (Table 29a and Fig. 8 and 9).

Takge *et al.* (1964) and Iwasaki *et al.* (1967) found maximum milk clotting activity of the enzyme from *Mucor pusillus* at 56°C and animal rennet at 44°C. But the observations made by Yu *et al.* (1969) showed maximum milk clotting activity of the *M. pusillus* enzyme at pH 5.5 and 70°C and it did not clot milk at pH 7.0.

Shovers and Baviotto (1967) observed peak milk clotting activity of *F. parasitica* rennet at 57.5°C and of animal rennet at 55°C. But Sardinae (1972) reported that the enzyme had maximum activity at 62 to 63°C.

An enzyme of *R. fulva* showed maximum activity at 64 - 66°C and at a pH similar to animal rennet (Rapo *et al.*, 1969 and 1970). Kawai (1970) observed that a temperature of 55 to 60°C was optimum for the milk clotting activity of Basidiomycete enzymes. He also showed that maximum milk clotting activity was exhibited at pH 5.4. The milk clotting enzymes of *Iroreia lactea* and *Gastrula rennera* were influenced by temperature changes in a similar manner to that of animal rennet. The enzyme of *Fomitopsis pinicola* was influenced to
to a greater extent by temperature changes.

(ii) Curd Tension

The curd formed by A. subtilis enzyme was softer than that formed by animal rennet but was harder than that formed by Meito rennet (Table 30). With the addition of 0.1% CaCl₂ to the substrate the firmness increased relatively to a greater extent with Meito and animal rennets than with A. subtilis rennet.

Krishnaswamy et al. (1973) observed that mild and bacterial rennets formed softer curd than animal rennet. The enzymes of A. nidulans used in their study formed a curd which was more firm than bacterial rennet curd prepared using enzymes of A. oryzae, Str. liquefaciens and A. subtilis.

Kikuchi et al. (1988) found that the curd tension increased or decreased with the holding time of the milk-enzyme complex as well as with the heat treatment given to the substrate. The above observations suggest that in the case of both A. subtilis and Meito rennets a longer holding period of curd is needed to obtain a curd with satisfactory firmness.

Teugo et al. (1964) had made similar observations with Meito rennet. They noted that the curd formation strength of Meito rennet was lower than that of animal rennet. With the addition of 0.01 to 0.02% calcium chloride they obtained a higher restoration of the curd formation property than animal rennet.
(iii) Influence of calcium chloride on the milk clotting activity

The milk clotting activity of Absidia rennet increased by 7.9 folds when 0.2% calcium chloride was added to skim milk substrates. This increase was almost similar to that obtained with animal rennet (8.04 folds). The increase in the activity of Meite rennet (19.32 folds) was more than both Absidia and animal rennets (Table 31 and Fig. 10).

The milk clotting activity of Ruscor rennet was observed to be influenced to a greater extent by the addition of calcium chloride than animal rennet by a number of workers (Tsuge et al., 1964; Iwasaki et al., 1967, and Richardson et al., 1967).

Sardinas (1967) and Shovvers and Devinette (1967) also observed similar influence of Ca ions on the milk clotting properties of Endothia rennet and animal rennet. Calcium chloride was reported to influence the milk clotting activity of Ruscor michaei rennet to a greater extent than animal rennet (Pederson, 1969). Similarly the milk clotting enzyme of F. pinicola was influenced to a greater extent by the addition of CaCl₂ than animal rennet, whereas the enzymes of Lactobacillus casei and Lactobacillus lactis were similar to animal rennet (Kawai, 1970).

These results indicate the individual variations in the milk clotting mechanism of different rennets, as Ca is one of the essential factor responsible for the clot formation and firmness of the curd.
(iv) Influence of activators and inhibitors on the milk clotting activity

In order to get an insight into the nature of the enzyme, its cofactor requirements and the nature of the active centre, activators and inhibitors are employed in enzyme studies. The studies also help to compare the properties of an enzyme with those of other enzymes. The present investigation with Absidia rennet has given useful and interesting information in this regard.

The milk clotting activity of *A. reeves* was inhibited by $10^{-3}$ M concentration of AgNO$_3$, NiSO$_4$ and KMnO$_4$ (Table 32). The activity was not much affected by reducing agents, sulphydryl inhibitors and heavy metal ions or 2-mercaptoethanol. The enzyme showed increased activity when treated with $10^{-2}$ M EDTA.

In respect of the above properties Absidia enzyme showed certain similarities and certain differences with animal and other fungal rennets. The inhibition of Absidia rennet by KMnO$_4$ which is an oxidising agent shows the presence of oxidizable amino acids in the active centre. The enzymes of *M. pusillus* (Iwaseki *et al.*, 1967) and *Pen. duboniti* (Hashimoto *et al.*, 1973) were also reported to be inhibited by KMnO$_4$. The enzyme of latter organism was also inhibited by another oxidizing agent N-bromosuccinimide. Yu *et al.* (1971) in studies with oxidizing agents, photooxidation and reaction with Diazo–H-tetrazole have concluded that histidine is present in the active centre of the milk clotting enzyme of *M. pusillus* Lindt.

For finding the active centre of Absidia rennet similar investigations with other specific inhibitors are necessary.
The increase in activity of milk clotting enzyme of *A. remora* in the presence of EDTA, which is a chelating agent suggests binding of certain metallic ions by it. These metallic ions may be present along with the enzyme limiting its activity. The inhibition of the proteolytic activity to certain extent suggests the probability of this metallic ion being associated with activation of the proteolytic component of the enzyme.

Kawai (1970) has shown that milk clotting enzymes of Basidiomycetes and animal rennet are inhibited by EDTA but activated by MnCl₂, MgCl₂ and Cysteine. The enzyme of *A. michi* was reported to be activated by EDTA, MnCl₂, CaCl₂, NH₄Cl and Diisopropyl fluorophosphate (Sternberg, 1971).

The enzyme of *A. remora* was not inhibited by sulphydryl inhibitors like Mercaptoethanol and Thiomercaptoal as was the case with *R. pusillus* (Iwasaki *et al.*, 1967) and Endothia rennet (Hagemeyer *et al.*, 1968). In this respect Absidia enzyme appears to be similar to most of the milk clotting enzymes in not having any sulphydryl group in the active centre.

While a heavy metal ion like HgCl₂ was not much inhibitory to Absidia rennet, it completely inhibited *A. michi* rennet (Sternberg, 1971) and *R. pusillus* rennet (Iwasaki *et al.*, 1967).

The Ferric ions had only slight inhibition on the milk clotting activity of Absidia rennet and resembled *R. pusillus* enzyme (Iwasaki *et al.*, 1967) in this respect but differed from the enzyme of *Rhizopus chinensis* (Taura *et al.*, 1978) the activity of which was completely inhibited by this ion.
Those chemicals which inhibited milk clotting activity did not always inhibit the proteolytic activity of A. rennet. MgCl₂, NiSO₄, SnCl₂ and Cysteine were inhibitory to certain extent to the milk clotting activity of Absidia rennet but they either showed no effect on proteolytic activity or stimulated it to certain extent. Sternberg (1971) reported that $\text{Al}_2(\text{SO}_4)_3$ which inhibited the milk clotting activity of the enzyme of M. miehei increased the proteolytic activity of the enzyme. These results indicate the probability that the proteolytic component and milk clotting component are two different enzymes.

(v) Influence of phosphate buffers

Studies conducted with phosphate buffers indicated that they were inhibitory to the clotting activity of Absidia rennet (Table 33). This is probably due to the binding of Ca⁺⁺ which is necessary for milk clotting activity by the phosphate group. These studies also show that Ca ion is essential for coagulation of esselin by this enzyme as in animal rennet.

(vi) Heat stability of the enzyme

Liquid enzyme preparation of Absidia rennet was stable for more than one hour at 50°C and only 40 per cent of its activity was lost in 15 minutes at 60°C (Table 34).

These results show that Absidia rennet has higher heat stability than animal rennet. Similar observations have been made by Teuge et al. (1964) who reported that animal rennet lost 35% or more of its activity at 50°C within 60 minutes while Maita rennet showed a
loss of only 20 per cent. Those results also indicate that Absidia rennet is more heat stable than Maito rennet.

Absidia enzyme seems to be more heat stable than enzymes of *Aspergillus candidus*, *Aspergillus nidulans* and *Endothia parasitica* as most of these enzymes have been shown to get completely inactivated at 60°C within 5 minutes (Veselov et al., 1965; Krishnamurthy et al., 1973 and Sardines, 1967). But this mold enzyme was less heat stable than the enzyme of *A. cerevisiae* as it is reported to be stable at 70°C for 3 minutes by Oosthuizen (1962).

(vii) pH stability

Absidia rennet was stable for 3 hours over a wide pH range of 2.0 to 7.5 at 40°C. At pH 4.0 the total enzyme activity was more than the control even though there was reduction.

Similar studies conducted by Nickelson and Ernstson (1967) and Arima et al. (1970) have shown that the enzyme of *Rhizopus chinensis* was stable between pH 3 - 6. Hagemeyer et al. (1968) reported that *Endothia rennet* was stable between a narrow pH range of 4 and 5.

Teuru et al. (1970) found that the acid protease of *Rhizopus chinensis* was stable between pH 2.8 and 6.5. The enzymes of *Ipomex lacteum* and *F. pinicola* were stable between pH 3 and 5 and enzyme of *Coriolus versicolor* was stable between pH 3 and 4 (Keisai, 1970). Acid protease from *Rugor michaei* was stable between pH 3.0 and 6.0 (Ottessen and Rickert, 1970). Kim et al. (1971) reported that the enzyme of *Dendripora ribis* was stable between pH 6.0 and 8.0. Milk clotting enzyme of *Sarcoscypha fulva* was stable between pH 3.0 and 6.85 (Chu et al., 1973).
These observations indicate that Absidia rennet has a higher stability in acidic pH like other mold enzymes except Pithiotrella ribis.

(viii) storage stability

Nearly 50 per cent of milk clotting activity of Absidia rennet was lost by storing it in the liquid form at 10, 30 and 37°C, within 10 days while at these temperatures enzymes in the powder form did not show any loss of activity for 30 days (Table 36). Similar observations have been made by Tsugo et al. (1964) who observed that the powder form of Rucor pusillus rennet was more stable than enzyme solution. Ottosen and Rickert (1970) observed that the liquid form of animal rennet lost 40 per cent of activity at 25°C within 2 days while the enzyme solution of R. nigra with the pH ranging from 2 to 6 remained stable up to 8 days. Yu et al. (1969) observed that Rucor pusillus rennet was stable for 15 days at pH 5.0.

These results indicate that the loss of activity of Absidia rennet which has been sometimes observed is mostly due to the storage of the liquid enzyme without adjusting the pH. As the enzyme retained nearly 50% of activity for 10 days at 10, 30 and 37°C, it may be presumed to have longer shelf life than animal rennet.

(ix) Proteolytic activity on haemoglobin and casein

The optimum pH requirement for proteolytic degradation of haemoglobin by Absidia enzyme was pH 2.0, 4.0 and 7.0 and for casein it was between pH 4.0 and 5.5 with highest activity at pH 4.5 (Table 37 and Fig. 11). Slightly higher proteolytic activity on casein
was observed at pH 2.0 than at pH 2.5. The 3 peaks in activity observed with hemoglobin substrate indicate the presence of 3 types of proteases in this partially purified preparation. Among these three proteases, two seem to be acid proteases. Proteolytic activity was highest on hemoglobin at pH 2.0 than at pH 4.0 but on casein it was at pH 4.5 than at pH 2.0. This indicates that the enzyme fraction which is active at pH 2.0 is specific to hemoglobin. The enzymes which showed maximum proteolytic activity on casein at pH 4.5 showed considerably higher activity on hemoglobin also at pH 4.0. This obviously showed that this enzyme fraction was less specific. The fraction causing maximum proteolytic activity on casein, most probably, is the enzyme responsible for milk clotting activity. Further purification of the enzyme is very essential for confirming these results.

The milk clotting enzyme preparations obtained from *M. pusillus*, *M. michai*, *S. parasitica*, *S. fulva*, *Pen. duponti* and *Rhizopus chinensis* have been identified as acid proteases after purification (Sardinas, 1972).

The acid protease of *Mucor pusillus* was active on k-casein at pH 4.5 and on hemoglobin at pH 4.0 (Yu et al., 1969). *Mucor michai* acid protease was active on casein between pH 3.5 and 5.5 and on hemoglobin between pH 3.5 and 4.1 (Sternberg, 1971). Larson and Whitaker (1970a) found pH 2.0 and pH 2.5 to be the optimum for proteolytic activity by *Endothia* acid protease on hemoglobin and casein. *Penicillium duponti* acid protease was active on casein at pH 2.5 and on hemoglobin at pH 3.0 (Hashimoto et al., 1975b).
Rhizopus oryzae acid protease was most active at pH 2.9 to 3.3 (Tsaru et al., 1970).

These observations indicate the individual variations with most of the enzymes and on comparison Abesida rennet seems to be similar to the enzymes of *Hacor pusillus* or *Hacor alshai* in its pH requirements.

(x) Presence of other enzymes

The partially purified enzyme of *A. rennea* contained cellulase, amylase and lipase activities which were comparable to those present in Meito rennet. Animal rennet contained negligible quantity of amylase and had no cellulolytic or lipolytic activities (Table 38 and Fig. 12).

Presence of amylase activity has been reported earlier in the case of *H. pusillus* and animal rennets by de Koning et al. (1969). They also tested Endothia rennet and found it to be free from any amylase activity. These differences in various rennet preparations have been used for characterizing and grouping the different rennet substitutes. Abesida rennet falls in the same group as Meito rennet (*Hacor pusillus* rennet) as it has the same type of enzyme content, but differs from Endothia rennet or animal rennet due to its high amylase content.

(xi) Diffusion in Casein Agar gel

Casein agar gel with pH 5.8 showed one precipitation and one clear zone with Abesida and Meito rennets and two precipitation zones and one clear zone with animal rennet (Fig. 13).
These findings are similar to the observations made by Ganguli and Bhalerao (1945) who found one precipitation and one clear zone with bacterial rennet and two precipitation and one clear zone with animal rennet.

de Koning et al. (1969) used this technique for studying the behaviour of different milk clotting enzymes. They observed one zone of precipitation and one clear zone with all the milk clotting enzymes obtained from microbial sources. Only animal rennet showed two zones of precipitation and one clear zone. Richardson (1970) also made similar observations with animal and microbial rennets.

With casein agar gels adjusted to pH 6.5 one precipitation zone was observed with animal rennet while Absidia and Reito rennets showed the presence of one precipitation zone and one clear zone. An interesting observation was also made in these gels. The points at which animal and Absidia rennets met a straight band of precipitation was observed (Fig. 14). It will be very interesting to study the nature of this band, as well as the reaction which is responsible for this precipitation. This type of precipitation was not observed between animal and Reito rennet or Reito and Absidia rennets.

This precipitation band at pH 6.5 between Absidia and animal rennets may be due to the activation of an inactive component of animal or Absidia rennet at the point of contact and subsequent precipitation of casein. (Preliminary work carried out by mixing the two enzyme preparations in equal volumes have shown a synergistic effect on the milk clotting activity which increased by more than 40 per cent of the sum total).
(xii) Identification of Rennet Substitutes

For identification of rennet substitutes several methods have been used by different workers. These have been discussed in detail by de Koning (1972) and de Koning and Draaisma (1973).

According to them following techniques are used in identification.

a) Casein agar diffusion.
b) Starch agar diffusion.
c) Electrophoretic mobility of different enzyme preparations.
d) Gel filtration and column chromatography.
e) Immunological identification.
f) Inhibition of clotting activity.
g) Inhibition of Microbial lipase.
h) Differentiation by their proteolytic activity.
i) Identification by isoelectric focusing.

Among these techniques Casein agar diffusion (Fig. 13), starch agar diffusion (Fig. 12) and electrophoretic patterns of cheddar using different enzymes have been used for differentiation of Absidia rennet from animal and Meito rennets (Fig. 18 and 19) in the present study. In addition, testing for the presence of other enzymes and polyacrylamide-SDS gel electrophoresis were also carried out to differentiate the three rennets.

The results of polyacrylamide-SDS gel electrophoresis are diagramatically represented in Fig. 15. This technique is also used for
estimation of molecular weights and from the similar mobilities shown by the major bands of Absidia and animal rennets it can be assumed that they have similar molecular weights. Meito rennet with faster mobility has a different molecular weight. The molecular weight is inversely proportional to electrophoretic mobility in this technique. The molecular weight of Rupor musillus acid protease has been reported to be 29000 to 30000 and that of animal rennet 31000 to 40000 by Yu et al (1969). From this it can be assumed that Absidia milk clotting enzyme has also a molecular weight between 31000 to 40000.

Although this technique cannot be used for differentiation of Absidia and animal rennets, because of their similar mobilities it can be used to differentiate Absidia rennet from Meito rennet.

This technique will be useful in the differentiation of Absidia enzyme from Meito rennet when used along with casein agar diffusion or starch agar diffusion techniques wherein these two enzymes show similar characteristics. With these three tests it will be possible to differentiate Endothia rennet also as it has no amylase activity in the preparation (de Koning et al, 1969).
Presence of Aflatoxin and Inhibitory Substances

The extract of the wheat bran culture of *A. remote* did not show the presence of aflatoxin when tested by T.L.C. technique. There were no fluorescent spots in the plate at the place where the sample had been run. Testing for the presence of any other toxin, needs laboratory animals as done by Legten et al. (1972).

From the results on acidity development during cheddar- ing (Table 39) it was evident that it did not produce any adverse effect on the growth of starter. Similar trials conducted by Havlova and Dolazalek (1973) have shown that microbial rennets and animal rennets inhibited the growth of Emmental cheese starters but not other starter cultures. They found that the milk clotting enzymes stimulated the starter cultures slightly during acid development.
VII. Preparation of Cheddar cheese

(1) Changes during manufacture

(a) Amount of rennet needed: About 8 g of Absidia rennet with 8000 units of milk clotting activity was required to clot 100 litres of milk for the cheese preparation. This was equivalent in activity to 2-3 g of commercial Meito rennet powder or 5 g of Hansen’s rennet powder (Table 39). The time taken for clotting by Absidia rennet varied from 30 to 45 minutes while it was 20-25 minutes with Meito rennet and 45 minutes with animal rennet. The type of curd formed was similar.

The amount of microbial and animal rennets used by different workers for cheese making have differed considerably. Kikuchi et al. (1968a) used 10 g of P. polymyxna rennet, 4.7 g of Meito rennet and 2.8 g of animal rennet for preparing Edam cheese. Propotnik (1968) observed that 0.7 g of Rucor rennet was sufficient for curdling 100 kg milk with 20 g CaCl₂ in 35 minutes while 2.2 g of Endothia rennet was needed for 100 kg milk with 10 g CaCl₂.

It had been noted earlier that with the adjustment of pH of the enzyme solution to 4.0 using citrate buffer (Table 35) the milk clotting activity increased by four folds. If the pH of the enzyme is adjusted to 4.0 then the quantity of enzyme needed can be reduced to 1/4th of the original quantity. Investigations on these lines will be useful for economical utilization of this enzyme. This may also help to reduce the proteolytic degradation in the initial stages as the proteolytic activity of the enzyme is only slightly influenced
with the adjustment of pH.

(b) Acidity development during manufacture: The acidity development during cheddaring with Absidia rennet was slightly faster than Reito rennet and similar to animal rennet. No differences were observed in the case of animal, Absidia or Reito rennet in the curd (Table 39).

These results indicate that Absidia rennet neither inhibits nor excessively stimulates the starter culture.

(c) Fat loss in whey: Mold enzymes showed slightly higher fat loss in whey than animal rennet cheese whey. The fat losses in cheese whey was similar with Reito and Absidia rennets (Table 39). Kikuchi et al (1968) observed higher fat loss in whey when \( \textit{M. pusillus} \) enzyme was used for Cheddar cheese making. Zweginga et al (1969) did not find any difference in the whey composition between cheeses from \( \textit{Mucor pusillus} \) rennet and animal rennet. The results of the present investigation agree with the findings of Antila and Apola (1969), who observed higher fat loss in whey with \( \textit{M. pusillus} \) rennet.

(d) Yield of cheese: The yield of Absidia rennet cheese was 10.4 to 11.9 kgs which was less than that of animal rennet cheese (12.5 kg) but was similar to Reito rennet cheese (10.5 to 10.8 kg).

The lower yield may be due to higher loss of protein in whey with microbial rennets. The moisture content of 5 days old Absidia rennet cheese was also lower than Reito and animal rennet cheeses.
indicating an additional cause for the lower yield. Kikusui et al. (1968) observed higher protein loss in whey with \textit{M. pusillus} rennet. The dry matter and fat loss were also higher with this enzyme. Fukumoto et al. (1970) also observed similar loss of proteins in whey with animal rennet and \textit{Rhizopus nivens} enzyme.

Although higher proteolytic breakdown was observed with Maito rennet, Tsuge et al. (1964) obtained similar yields of cheese with Maito and animal rennetes. They advised the addition of calcium chloride to get better yield in the Gouda cheese.

Lower yields of cheese have been reported by a few other workers with the following mold enzymes: \textit{M. pusillus} rennet (Robertson and Gilles, 1969); \textit{Mucor miehei} rennet (Aames, 1971); Endothia rennet (Meubois and Macquot, 1969 and Edelsten et al., 1969).

Higher yields of cheese have also been reported with Maito rennet and \textit{M. miehei} rennet (Edelsten et al., 1969; Romet and Aaleis, 1972). The addition of calcium chloride before renneting has been suggested for obtaining higher yield (Prins, 1973).

With the above observations it can be concluded that Absidia enzyme compares favourably with other mold enzymes and can be used with the following modifications in the manufacturing techniques:

1) Addition of CaCl$_2$ to milk during renneting.

2) Adjustment of pH of enzyme to 4.0 thereby reducing the quantity of enzyme needed for clotting and also minimising protein degradation.
(ii) Changes during ripening

(a) Moisture content: During the six months ripening the decrease in the moisture content of the Absidia rennet cheese was comparable to that of animal rennet cheese but was higher than Reito rennet cheese (Table 40).

According to Edelsten et al (1969) the Domati cheese prepared with Reito rennet showed an increase in the moisture content after three months of ripening. The cheese was greasy.

Microbial rennet cheeses with moisture content similar to animal rennet cheeses were obtained with Reito rennet in Edam, Gouda and Cheddar cheeses (Kikuchi et al, 1968), Gouda and Camembert cheeses (Tsugo et al, 1964) and with Endothia rennet in Edam, Tilsit and Butter cheeses (Thomasew et al, 1970). Kikuchi and Toyoda (1969) observed higher moisture contents in cheeses prepared using crystalline enzymes than commercial preparations.

(b) Fat content of cheeses: The cheeses with Absidia rennet showed a fat per cent of 29.0 to 29.5 after six months of ripening. Animal rennet cheese showed a fat per cent of 28.5 and Reito 24.0 to 26.5. The lower percentage of fat in the Reito rennet cheese is most probably due to its higher moisture level at six months ripening. If the fat in cheese is calculated on the dry weight basis then with Absidia rennet, animal rennet and Reito rennet cheeses it would be 44.39 to 46.09%, 45.72%, 39.54 to 42.88% respectively (Table 40).
Other microbial enzymes like Endothia rennet, Maite rennet, Suparen, Newry rennet and renilase have also been reported to yield cheese varieties like Edam, Tileit and Butter with similar fat content as found in animal rennet cheese (Thomasow et al., 1970; Nelson 1969 and Asspale et al., 1972).

(c) Changes in pH and acidity: There was a gradual rise in pH and fall in acidity with all the three batches of cheese during six months ripening. During the first month there was a little increase in the acidity which subsequently decreased. The change was considerable between one and six months ripening period. There was no difference in pH between the cheeses prepared using Absidia rennet, Maite rennet or animal rennet (Table 41).

The increase in acidity at the end of one month ripening was higher in Absidia rennet cheese than Maite rennet cheese or animal rennet cheese. Even in six month old cheeses the acidity was higher with Absidia rennet cheeses.

These observations show similarities in the trend of acid formation and pH changes with those observed earlier by Teugo et al. (1964), Kikuchi et al. (1968, 1969) and Kikuchi and Toyoda (1969), with Maite and Hansen’s rennets in the preparation of Gouda, Cheddar and Edam cheese types.

(d) Nitrogen content: Total nitrogen content of all the three rennet cheeses did not show much variation during the six months ripening, while there was a steady increase in sodium citrate-HCl
soluble nitrogen with all three rennet cheeses during this period.

Abaidia rennet cheese showed higher quantity of sodium citrate-HCl soluble nitrogen than Maite rennet cheese or animal rennet cheese. The rate of increase in sodium citrate-HCl soluble nitrogen was, however, highest with Maite rennet cheese, followed by animal rennet cheese and Abaidia rennet cheese (Table 42).

The maturity index as calculated by the following formula

\[
\frac{\text{soluble nitrogen}}{\text{Total nitrogen}} \times 100
\]

showed values of 15.92 to 18.33 at 5 days ripening with Abaidia rennet cheeses and only 2.62 in animal rennet cheese and 2.93 to 8.30 with Maite rennet cheeses.

These results show that there is higher proteolytic breakdown initially with Abaidia rennet and not with Maite or animal rennets. But during ripening of cheese tertiary proteolysis showed a faster rate with Maite rennet and animal rennet than Abaidia rennet. The maturity index increased at a faster rate during the six months ripening period in the following order - Abaidia rennet < Animal rennet < Maite rennet. At the end of six months the maturity index was 34.92 in Maite rennet cheeses, 23.9 to 30.5 with Abaidia rennet cheeses and 19.23 with animal rennet cheese (Table 43 and Fig. 16).

The observations made above are similar to those made by Kikuchi et al (1968) in the Edam, Gouda and Cheddar cheese preparation with Maite and Hansen’s rennets.
Tsugo et al. (1964) observed lower quantities of water soluble protein nitrogen in Malta rennet than animal rennet. The reason for this lower value was attributed to the release of larger non-casein nitrogen by Malta rennet.

Higher rates of increase in the maturity index with microbial enzymes as compared to animal rennet have been observed in the case of Malta rennet (Tsugo et al., 1964; Kikuchi and Toyoda, 1969) and Endothia rennet (Edelsten et al., 1969). Amongst the microbial rennetes Endothia rennet showed higher rate of increase in maturity index than Malta or R. miehei rennetes, and Malta rennet than R. miehei rennet, the last one showing the slowest rate with the Egyptian variety of cheese (Edelsten et al., 1969).

The results of present study indicated that the enzyme of Absidia was more close to animal or R. miehei rennet in its tertiary proteolysis than Endothia or Malta rennet. The higher value of maturity index observed initially in Absidia rennet was but a reflection of its low ratio of milk clotting to proteolytic activity. This was obvious due to the fact that the enzyme preparation contained other proteolytic enzyme fractions which were active even at neutral pH. If these were separated from the milk clotting fraction then the higher loss of protein in whey during preparation could also be reduced.

(a) Changes in TCA soluble Tyrosines: There was approximately 3 fold increase in the 2.5 per cent TCA soluble tyrosine content in the case of Absidia and animal rennet cheese samples during six months ripening, while the increase with Malta rennet cheese was
The microbial rennet cheeses showed higher quantity of 12% and 2.5% TCA soluble tyrosine than animal rennet cheese. The quantity of 12% and 2.5% TCA soluble tyrosine was more with Absidia rennet cheese than Meito rennet cheese.

The increase in 12% TCA soluble tyrosine was by 2.7 to 3.8 folds over the initial value with Absidia rennet cheeses, 4.4 times with animal rennet cheese and 4.5 to 4.9 times with Meito rennet cheeses after six months ripening (Table 44 and Fig. 17).

These results confirmed that tertiary proteolysis was slower with Absidia rennet than Meito or animal rennet cheeses. Out of the three rennets Meito showed the fastest rate of tertiary proteolysis.

In Absidia and Meito rennet cheeses nearly 90 per cent of 2.8% TCA soluble tyrosine was in 12.0% TCA soluble form when the cheese was six months old. This was only sixty per cent with animal rennet. These results of TCA soluble tyrosine were directly proportional to nitrogen content.

Kikuchi et al. (1968) obtained similar results earlier with Meito rennet and animal rennet during the ripening of Edam, Gouda and Cheddar cheeses.

The results on the degradation of proteins showed that the ripening of cheese by microbial rennets was faster than by animal rennet.
clear cut differences in starch gel electrophoretic characteristics (Fig. 18). While Absidia rennet cheese showed the degradation of both $\alpha$- and $\beta$-casein fraction, animal rennet cheese showed the degradation of $\alpha$-casein only. Heito rennet cheese showed complete degradation of $\alpha$-casein and partially $\beta$-casein. With this 8.6 pH gel there were two positively charged bands in the case of animal rennet cheese while in Heito rennet cheese there was one band only and in Absidia rennet cheese there were none. Comparatively Absidia rennet showed higher proteolytic breakdown than animal or Heito rennet.

The observations made with animal rennet and Heito rennet cheeses agree with the observations made by Itoh and Thomasew (1971).

Nickelson and Fish (1970) also observed differences in the electrophoretic patterns in casein digested with different milk clotting enzymes. They have observed higher proteolytic breakdown of whole casein with Endothia rennet than animal or Heito rennets. This enzyme digested $\beta$-casein also to a great extent when compared with animal and Heito rennets.

The degradation of mainly $\alpha$-casein and to less extent $\beta$-casein by rennin has been reported earlier by Ledford et al (1968) and El-Megoumy (1968). Similar results have been obtained in the present investigation.

From the above observations it is evident that Absidia rennet is similar to Endothia rennet than animal and Heito rennets in its
proteolytic activities on casein.

Polyacrylamide gel electrophoresis also showed certain differences in cheeses prepared using Absidia, animal and Meito rennets. Absidia rennet cheeses showed seven bands while animal and Meito rennet cheeses showed only six bands. The electrophoretic patterns of slower moving six components of Absidia rennet cheese were similar to animal rennet cheese components. Five bands which moved faster were similar in Meito and animal rennet cheese fractions. One slow moving band of Meito rennet showed lesser mobility than the sixth band of animal rennet cheese (fig. 19).

These results obtained with Meito rennet and animal rennet cheeses were not similar to the observations made by Weckx and Vanderpoorten (1972) in Gouda cheese. But it confirmed the applicability of this technique in the detection of the source of rennet used for cheese preparation. The difference observed in the present investigation and those made by Weckx and Vanderpoorten (ibid) may be due to variations in cheese manufacturing technique, the type of starter used and the age of cheese.

The relative specificity of Absidia rennet on casein fractions can be known only after testing the rate of proteolysis by this enzyme on different fractions.

(g) Organoleptic quality of cheese: The cheeses made with Absidia rennet showed slight bitter taste during the initial stages of ripening. This disappeared after a month's ripening. There was
no bitterness in the animal or Malta rennet cheeses at any stage of ripening.

The other qualities of cheeses like body, texture, consistency, flavour and acceptability were similar in all the three types of rennet cheeses. On the basis of scoring given in Table 45 Absidia rennet cheese was found superior to animal rennet or Malta rennet cheese.

The disappearance of bitterness after one month ripening indicated that there was liberation of bitter peptides during early stages of ripening due to higher proteolysis and that further breakdown of the peptides had occurred during further ripening. It may be due to the enzymes liberated by starter culture or due to the Absidia enzyme itself. The study of the release and breakdown of these bitter peptides and the enzyme responsible for it will be useful in separating this component from the enzyme preparation. Study of suitability of different starters may also help in overcoming this defect.

Vos (1942) reported bitterness with cheese prepared using an enzyme preparation from *Stx. liquefaciens*. But the enzyme obtained from another strain of the same species gave cheese without any bitterness (Srinivasan *et al*, 1968). Kimuchi *et al* (1968) observed persistent bitterness with *R. polymyxa* rennet when it was used for Gouda, Cheddar or Edam cheese preparation. But the cheese prepared by Isai *et al* (1970) using the same bacterial enzyme did not show any bitterness. Puhan (1967) used the enzyme of *R. subtilis*
in cheese manufacture with slight modification in the manufacturing technique and obtained cheese with a lower score than animal rennet cheese.

Chebotarev et al. (1966 and 1969) obtained good quality Bryndza and Doraougbuch cheese using an enzyme of Asp. candidus. The enzyme of Rhizopus chinensis has also been reported to give good quality Cheddar cheese (Fukumoto, 1970). Kawai and Mukai (1970) obtained good quality Cheddar cheese using an enzyme preparation from Irinex lacteus. But when they used an enzyme preparation from Fonitepsis pinicola a bitter cheese was obtained.

Several types of cheeses have been prepared using commercial rennet preparations obtained from Musor pusillus, R. miehei and E. parasitica. Most of the reports state the suitability of these enzymes in cheese preparations. Except in a few cases where the cheese blocks were noticed to develop bitterness, the cheeses prepared using these enzymes were reported to be of good quality.

Different varieties of cheeses of satisfactory quality have been prepared with Maito rennet e.g. Cheddar, Brick, Butter, Camembert, Cottage, Edam, Gouda, Italian varieties and Tilsit (Arise et al., 1970; Richardson et al., 1967; Robertson and Gilles, 1969; Schulz et al., 1967). Development of bitterness and body defects were noted in some of the cheese blocks prepared with this enzyme (Babel and Komuti, 1968; Kikuchi and Toyoda, 1969; Kikuchi et al., 1968 a,b). On the other hand it has been shown by Kikuchi et al. (1968 a), Pederson (1969) and Yu et al. (1971) that by modifying the cheese making procedure or by purifying the enzyme acceptable
quality cheese may be produced.

Behnke and Siewert (1969) and Handy (1972) obtained satisfactory quality Edam, Camembert and Domiati cheeses using *Mucor miehei* rennet. Prina and Nielsen (1970) prepared good quality Cheddar cheese with this rennet. Thomasaw (1971) observed accelerated curing of Edam, Tileit and Buttermilk cheeses with *M. miehei* rennet. Martens (1973) noted the development of bitterness in Gouda cheese prepared using this enzyme. Prina (1973) suggested certain modifications in the manufacturing techniques for producing better quality cheese using this enzyme.

Many types of cheeses have been successfully prepared with Endothia rennet. Some of them have been found superior to those prepared with animal rennets. The types of cheeses with satisfactory quality prepared using this enzyme are Brie, Camembert, Cheddar, Colby, Edam, Emmentaler, Gruyere, Italian varieties, Limberger, Monterey, Munster, Teloggio and Swiss (Puhan and Staffan, 1967; Shovers and Savinette, 1967; Bolliger and Schilt, 1969; Ramet et al., 1969; Ramet and Schluter, 1970; Remini et al., 1971; Nadasdy, 1972). Edelsten et al. (1969) found that Domiati cheese prepared with this enzyme was greasy with higher moisture content and had bitter taste. Rezis and McKenzie (1970) produced good Cheddar cheese using 1:1 mixture of Endothia and animal rennets.

These observations made by different workers using various rennets indicate the suitability of microbial preparations with minor adjustments in the manufacturing techniques or purification of the enzymes.
The results of the present study indicate that Abaidia rennet can be used as a satisfactory rennet substitute. It was better than Meito rennet as it behaved like animal rennet under the influence of calcium and the curd formed with this enzyme was firmer than that formed by Meito rennet. It had better heat stability and storage stability than animal rennet and was not inhibitory to starter culture. The yield of cheese made with Abaidia rennet was comparable to that made with Meito rennet. The period of ripening with this enzyme preparation was less than that required for animal and Meito rennet preparations as it had higher proteolytic activity than the two enzymes. The Abaidia rennet, however, gave cheese having better organoleptic quality.

It can be easily identified by polyacrylamide-SDS gel electrophoresis, casein agar diffusion and amylase activity tests.