SUMMARY

Studies on Milk Clotting Enzyme from Rôlea
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1) A total number of 90 mold isolates were screened for the production of milk clotting enzymes among which 29 showed presence of the enzyme. One isolate WBC-1 identified as Absidia ramosa was selected for the study on the basis of high ratio of milk clotting to proteolytic activity shown by its enzyme.

2) *Absidia ramosa* produced maximum amount of milk clotting enzyme on semisolid wheat bran medium and very negligible concentration in liquid media.

3) Maximum concentration of the enzyme was obtained when the mold was grown on semisolid wheat bran medium with 50 per cent moisture, at a pH range of 3 to 8 and an incubation temperature of 22°C for a period of 96 hours.

   By employing two consecutive incubation temperatures, the first at 30°C for 24 hours followed by the second at 22°C for 48 hours, it was possible to get either similar or higher concentration of the enzyme, thus reducing the period of incubation by 24 hours.

4) Supplementation of 2.0% dextrose, 2.0% molasses, 0.2% sodium chloride, 0.5% protease peptone, peptone or tryptone, 25.0% skim milk,
1.0% malt extract or 1.0% (NH₄)₂SO₄ individually to semisolid wheat bran medium resulted in an increase in the milk clotting enzyme production by the mold.

5) Supplementation of wheat bran medium with 0.25% starch, 0.1% Mg-citrate, 1.0% NaNO₃, 1.0% NH₄NO₃ or 2.0% corn steep liquor resulted in a decrease in the enzyme production.

6) Complete extraction of the enzyme from the wheat bran culture was obtained by shaking the bran with 10 volumes of distilled water (w/v) for one hour at room temperature or by freezing the culture with similar quantity of water overnight and then thawing.

7) The crude enzyme extract was concentrated to 1/15th of its original volume by vacuum evaporation at 40°C without any loss in activity.

8) The crude enzyme extract was purified by 10.45 folds by precipitation with ammonium sulphate (60.0% w/v), acetone (1 : 1.5 v/v) and gel filtration through Sephadex G-75 column with a recovery of 68.8% of milk clotting activity. Gel filtration showed five protein peaks of which two contained milk clotting activity.

9) For the precipitation of concentrated enzyme only 50 per cent (w/v) of ammonium sulphate was needed and this precipitation gave a recovery of 68.65 per cent. Subsequent precipitation of the redissolved precipitate with acetone (1 : 1.5 v/v) or isopropanol (1 : 1 v/v) did not show any loss in milk clotting activity.

10) The optimum temperature required for bringing about clotting of milk was 55°C with Absidia and animal rennets while it was 60°C with
Maiko rennet. Maximum milk clotting activity was observed at pH 5.8 with milk as substrate in the case of all the three enzymes (Absidia, Maiko and animal rennets).

11) The milk clotting activity of Absidia and Maiko rennets were influenced to a greater extent by changes in temperature and pH than animal rennet.

12) The curd formed by Absidia rennet was more firm than that formed by animal rennet.

13) The influence of calcium chloride on the milk clotting activity was more on Maiko rennet than on Absidia or animal rennets. Absidia and animal rennets showed similar increase in activity with the increasing concentration of calcium chloride in the milk.

14) The milk clotting activity of Absidia rennet was inhibited by more than 70% in the presence of $10^{-3}$ M KI, $\text{HgSO}_4$, $\text{HgNO}_3$, or $\text{AgNO}_3$.

It was not much affected by the presence of sulphhydryl inhibiting agents indicating the absence of sulphhydryl group in the active centre of the enzyme.

15) EDTA at $10^{-2}$ M concentration increased the milk clotting activity of Absidia rennet by 25%.

16) Phosphate buffers used as enzyme diluents were inhibitory to the milk clotting activity of the enzyme.

17) In the liquid form Absidia rennet was stable at 50°C for more than one hour and lost 60% of its activity at 60°C within 15 minutes.
At 40°C and at a pH range of 2.0 to 7.5, it was stable for more than 3 hours. In the powder form as ammonium sulphate precipitate it was stable for 30 days at 37°C.

10) When the enzyme solution was adjusted to pH 4.0 the milk clotting activity increased by four folds.

19) Absidia rennet had maximum proteolytic activity on casein at pH 4.5 and on haemoglobin at pH 2.0 and 4.0 indicating that it is an acid protease.

20) The partially purified milk clotting enzyme preparation of *Absidia ramosa* contained amylase, lipase and cellulase activity like Haito rennet powder. Animal rennet had only negligible amylolytic activity.

21) While Absidia and Haito renneta gave one precipitated zone and one clear zone in casein agar gel with pH 5.8, animal rennet gave two precipitated zones and one clear zone.

In casein agar gel with pH 6.5 animal and Absidia renneta gave a precipitation band at the place of contact while no such reaction was observed between Haito and animal renneta or Absidia and Haito renneta.

22) In polyacrylamide–SDS gel electrophoresis the Absidia rennet showed five fractions, Haito rennet five fractions and animal rennet six fractions. The major fractions of Absidia and animal renneta showed similar electrophoretic mobilities while the major fractions of Haito rennet moved at a faster rate. On the basis of this it has been suggested that Absidia and animal renneta have similar molecular weights while Haito rennet has a lower molecular weight.
23) The yield of Cheddar cheese prepared using Absidia and Meito rennets was lower than the yield obtained with animal rennet.

24) The initial maturity index of Absidia rennet cheese was higher than animal or Meito rennet cheeses. The rate of increase in maturity index was more with Meito and animal rennet cheeses than Absidia rennet cheese during ripening.

25) Starch gel electrophoretic studies of cheese samples showed that animal rennet degraded mainly $\alpha$- casein while Absidia rennet degraded both $\alpha$- casein and $\beta$- casein. In the case of Meito rennet $\alpha$- casein was degraded to a greater extent than $\beta$- casein.

26) Slight bitterness observed in Absidia rennet cheese in the initial stages of ripening disappeared completely after one month. The body and texture of the cheese was comparable to those of animal and Meito rennet cheeses.

Absidia rennet cheese was found either superior or equal to animal and Meito rennet cheeses by organoleptic evaluation after six months of ripening.

27) On the basis of the above findings it was concluded that the enzyme of Absidia rennet could be used as a satisfactory rennet substitute in the preparation of Cheddar cheese.