1.0 ABSTRACT

Rennet is used in the manufacture of cheese from milk. As the calf rennet is expensive and scarce, microbial rennet (acid protease) has come into use. In this study microbial milk - clotting enzyme (acid protease) production by solid state fermentation (SSF) from *Aspergillus niger* N4 was studied using locally available substrates such as rice bran and soy flour.

A. niger was cultivated on basal medium mixed with paddy husk support in different ratios for acid protease production. Highest clotting [426 SU g Dry Mouldy Bran (DMB^{-1})] and proteolytic (14.2 PU g DMM^{-1}) activities were obtained at basal medium to paddy husk ratio of 10 : 0 and 3 : 7 respectively at 48 h. Maximum clotting (573.6 SU g DMB^{-1}) and proteolytic (8.8 PU g DMB^{-1}) activities were obtained at 48 h when the moisture content was 50%. Maximum clotting (437.6 SU g DMB^{-1}) and proteolytic (9.4 PU g DMB^{-1}) activities were obtained at 48 h when the initial spore density was 5 x 10^6 spores g $medium^{-1}$. However for large scale production of acid protease use of mycelial inoculum is more suitable. Hence continuous recycling of the mycelium as inoculum was studied. Mycelial inoculum of 12 h old and 5% (w/w) size was the best giving 620.5 SU g DMB^{-1} and 12.4 PU g DMB^{-1}.

The effect of supplementing basal medium with different nitrogen sources on acid protease production was studied to further improve acid protease production. When basal medium was supplemented with different inorganic nitrogen sources (at 1.15 g medium kg^{-1} elemental nitrogen level) at 48 h, NH_4NO_3 supplemented medium showed the highest clotting (114.6 SU g DMB^{-1}) and proteolytic (8.6 PU g DMB^{-1}) activities. Among the different organic nitrogen sources, soy flour supplemented medium gave the highest clotting (486.9 SU g DMB^{-1}) and proteolytic (14.6 PU g DMB^{-1}) activities at 48 h. From the above results, replacement of $(NH_4)_2SO_4$ with NH_4NO_3 in the medium

containing soy flour gave maximum clotting (582.2 SU g DMB^{-1}) and proteolytic (18.34 PU g DMB^{-1}) activities at 48 h. To the medium with NH_4NO_3 as inorganic source, different concentration of soy flour was mixed as the organic nitrogen source. Maximum clotting (600.9 SU g DMB^{-1}) and proteolytic (28.2 PU g DMB^{-1}) activities were obtained at 48 h in the medium containing 200 g soy flour and 3.0 g NH_4NO_3 $kg\ medium^{-1}$ and this medium was designated as production medium.

To the production medium, different inducers such as casein, gluten and egg albumin (20 g $kg\ medium^{-1}$) were added to improve the acid protease production. Addition of gluten gave maximum production of clotting (1410 SU g DMB^{-1}) and proteolytic (28.3 PU g DMB^{-1}) activities at 40 h. When different concentrations of gluten was added to the above medium, 10 g gluten $kg\ medium^{-1}$ gave the highest clotting (1718.4 SU g DMB^{-1}) and proteolytic (29.8 PU g DMB^{-1}) activities at 40 h. Since 10 g gluten $kg\ medium^{-1}$ supplementation (optimized medium) gave the highest enzyme activities, a study with the fixed amount of inducer (10 g $kg\ medium^{-1}$) and varying amounts of soy flour on acid protease production was performed. Increase in soy flour above 200 g $kg\ medium^{-1}$ decreased the clotting and proteolytic activities.

The above optimized medium was used to study the continuous production of acid protease with 5% (w/w), 12 h old mycelium as inoculum. Maximum clotting activity (3473.4 SU g DMB^{-1}) was obtained in the second batch that is with the first batch for mycelial inoculum. From the second batch (for mycelial inoculum) maximum clotting and proteolytic activities started to decrease gradually. However up to fifth cycle the activities obtained were nearly equal or more than that obtained with spore inoculum.

Stability of the acid protease enzyme in dry mouldy bran was studied. The enzyme showed stable clotting activity for about one month while the proteolytic activity remained constant only for one week.

Among the different extractants used to extract the enzyme from the dry mouldy bran, distilled water and 0.01M phosphate buffer (pH 6.5) were most suitable for the extraction of proteolytic activity while only 0.01M phosphate buffer (pH 6.5) was suitable for the clotting activity extraction. As the increase in clotting activity observed in the extractants was due to the ions, it was decided to extract the enzyme with distilled water. Maximum enzyme was extracted when fresh mouldy bran to distilled water ratio was 1 : 5. Since considerable amount of distilled water (10%) was retained by dry mouldy bran, extraction from dry mouldy bran was little lower than that from wet mouldy bran, but with the second extraction the recovery was 100%. A contact time of 45 min with agitation (150 rpm) improved the enzyme recovery.

The stability of acid protease was studied in buffer solutions with various pH values and at different temperatures. The enzyme was stable in the pH range from 2.5 to 4.0 for four days and two days with respect to clotting and proteolytic activities. The enzyme stored at 40°C or below were more stable than those at higher temperatures. At 50°C, 10% of the clotting activity was lost at 1 h, while at 60°C and 50°C respectively 80 and 21% of the proteolytic activity was lost at 15 min.

An attempt was made to reduce the proteolytic activity in the acid protease extract by adding calcium and / or zinc and incubating at 55°C and pH 4.5. Acid protease extract mixed with $ZnCl_2$ showed increase (66%) in clotting activity with no loss in clotting activity at 5 h incubation. However under the above conditions, acid protease lost 46% of the proteolytic activity. When $ZnCl_2$ was added to the supernatant of the acid protease extract and incubated for 4 h at 55°C, increase in the specific clotting and proteolytic activities of the acid protease from 15.4 to 33.4 and 0.7 to 1.5 respectively were observed.