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Production of fungal rennet by *Mucor miehei* using solid state fermentation

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Summary. Investigations have been carried out on the production of fungal rennet using a thermophilic strain of *Mucor miehei* under solid state fermentation conditions. A high milk clotting enzyme activity (58000 Soxhlet units/g) was achieved when optimum conditions were used. Further, a high ratio of 6.6:1 between milk clotting and proteolytic activities for this enzyme was obtained. Cheese prepared using this enzyme was also found to be acceptable in organoleptic quality. Large scale production of the enzyme in trays using the optimum conditions gave milk-clotting enzyme activities comparable to those in flask experiments.

Introduction

Calf rennet has been traditionally used for cheese-making all over the world. However, in recent years its shortage has necessitated a search for alternatives such as microbial production. Fungal rennets have received wide acceptability and in some countries 50%–60% of the market has been replaced by these substitutes for calf rennet (Birkkjaer and Johnk 1985; Crewford 1985). These enzymes are mainly produced by strains of *Mucor miehei*, *M. pusillus* and *Endothia parasitica*.

Solid state fermentation (SSF) is an economical and simple method for the production of industrial enzymes, traditional foods, toxins and some fine chemicals. Several fungi have been cultivated using the SSF technique for the production of rennet substitutes (Pozsar-Hajnal et al. 1974; Krishnaswamy et al. 1976; Yuan and Ochi 1986; Higashio and Yoshioka 1982).

Earlier work in this laboratory had resulted in the production of a satisfactory calf rennet substitute using the fungus *Rhizopus oligosporus* by SSF. However, the cost of the product was too high and a search was made for more potent microorganisms. A strain of *M. miehei* gave excellent results upon solid state cultivation. While some information exists on the cultivation of *M. miehei* by submerged fermentation, no information is available in the literature on protease production as a fungal rennet by this microorganism under SSF. Work was therefore undertaken to study the parameters influencing the production of the enzyme and the results are presented in this paper.

Materials and methods

Culture. The microorganism employed in this study was a thermophilic strain of *M. miehei* gifted by Professor P. Tauro of Haryana Agricultural University, Hissar, India. The stock culture was maintained on potato dextrose agar slants.

Acidic mineral salt solution (100 ml) was prepared containing (in g/l): $ZnSO_4 \cdot 7H_2O$, 0.07; $MgSO_4 \cdot 7H_2O$, 0.07; $CuSO_4 \cdot 7H_2O$, 0.07; $FeSO_4 \cdot 7H_2O$, 0.09; 0.2 *N* HCl. Ten millilitres of this solution was diluted to 1 l and 60 ml was used to moisten 100 g raw wheat bran (8%–10% moisture). One hundred grams of this moist wheat bran per flask was distributed in 1-l erlenmeyer flasks and autoclaved for 1 h at 15 psi. After cooling, the flasks were inoculated with a spore suspension prepared from *M. miehei* culture slants using sterile distilled water containing 0.1% Tween-80 and mixed thoroughly. Cultivation was carried out for 96 h in an incubator at different temperatures in the range 30°–50° C, with humidity controlled at 90%.

Large scale cultivation for enzyme production. The medium used for inoculum preparation described above was also used for large-scale cultivation of the culture. The standardised wheat bran medium with 70% moisture content was evenly dis-

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tributed in aluminium trays (16″ × 33″ × 12″) to a depth of 1″. Each tray containing about 2 kg moist bran was sterilized in an autoclave at 121° C for 60 min. The medium was inoculated (20% w/w) with the actively growing starter cultures of *M. miehei* and cultivation was carried out in a koji room at 42° ± 1° C with humidity controlled at about 90%. Fifty trays on each of two separate racks were loaded at a time for cultivation for the production of protease.

Extraction and recovery of the enzyme. After completion of fermentation the moist bran was dried at 55° C in a drier until the moisture content reached 12%–15%. A modified four-stage countercurrent extraction procedure involving solid liquid contact and pressing was employed to obtain a relatively high enzyme concentration in the product broth without further processing. The solvent used was tap water.

For preparation of liquid enzyme, the crude enzyme preparation was clarified in a Sharples (Surrey, UK) centrifuge and vacuum concentrated in a forced circulation evaporator. It was then stored at 8°–10° C after the addition of 0.1% sodium benzoate. This enzyme was not purified further. For preparation of powdered enzyme, the extract obtained was partially purified using absorbent clay, heat treatment and pH adjustment for eliminating enzymes such as lipases and non-specific proteases. This partially purified extract was treated with cold ethanol for precipitating the milk-clotting enzyme which was dried and powdered.

Assay of enzyme activities. Milk clotting activity was determined according to the method of Arima (1972) and expressed in terms of Soxhlet units (SU). One SU is defined as the amount of enzyme which clots 1 ml of a solution containing 0.1 g skim milk powder and 0.00147 g calcium chloride in 40 min at 35° C. The proteolytic activity was determined at pH 6.0 by the casein digestion method described by Kunitz (1947). Protein in the enzyme preparation was estimated by the use of Folin-Ciocalteau reagent according to the method of Lowry et al. (1951).

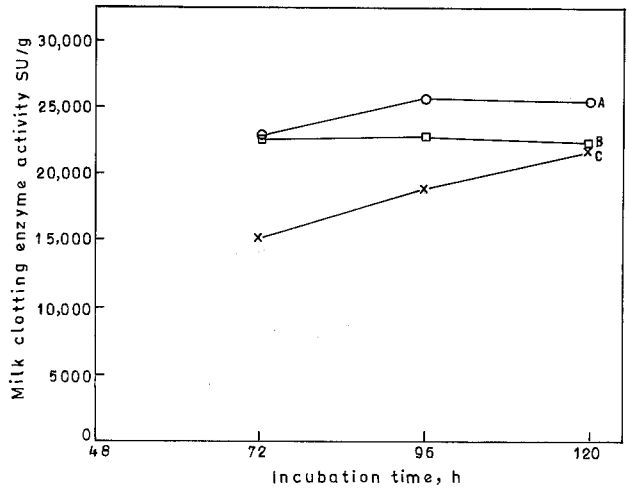

Fig. 1. Milk clotting enzyme production by *Mucor miehei* under solid state fermentation; SU = Soxhlet units. Carbon supplements: *A*, wheat flour; *B*, rice bran; *C*, rice flour

Results and discussion

Three different media were used for the initial experiments, all containing wheat bran as the main carbon source, but augmented (in the ratio 1:3) by different supplements (medium A, wheat flour; medium B, rice bran; medium C, rice flour). The cultivation was carried out at 37° C. The variation in milk clotting activity with time for the three media is shown in Fig. 1. Medium A was the best, giving an activity of 26000 (SU)/ml in 96 h.

Table 1. The comparison of *Mucor miehei* (Mm) rennet with commercial enzymes: milk clotting and proteolytic activities

Enzyme	Form	Milk clotting activity (Soxhlet unit)	Ratio of milk clotting to proteolytic activity
Rennilase	Liquid	112800/ml	3.9:1
Rennilase	Granular	660000/g	5.2:1
Noury rennet	Powder	800000/g	4.5:1
Sure curd	Powder	169000/g	1.6:1
Meito-rennet	Powder	662000/g	3.4:1
Hansen's rennet	Powder	363500/g	13.2:1
Calf rennet	Liquid	9600/ml	12.8:1
Mm rennet (cultivated on SSF[a])	Liquid	38400/ml	3.5:1
Mm rennet (SSF) after purification[b]	Liquid	67000/ml	6.7:1
Mm rennet (SSF) after purification[b] and precipitation	Solid	220000/g	6.6:1
Mm rennet, submerged fermentation	Liquid	1400/ml	0.63:1

[a] The cultivation medium for solid state fermentation (SSF) contained wheat bran:wheat flour (90:10); skim milk powder, 1%; moisture content, 70%; initial pH of mineral solution, 1.0; temperature for growth, 42° C

[b] Purification involves treatment with adsorbants, pH adjustment and heat treatment as referred to in Materials and methods

Since the ratio of milk clotting to proteolytic activity of rennets is an important factor for commercial cheese manufacture, this ratio for *M. miehei* protease cultivated under SSF conditions was compared with some of the commerical fungal rennets and the data are presented in Table 1. The *M. miehei* protease had a ratio of milk clotting to proteolytic activity similar to other commercial products.

However Table 1 also shows that *M. miehei*, when grown in submerged fermentation, produced more proteolytic enzyme than milk-clotting enzyme. This behaviour is similar to that of *M. pusillus*, which gave higher proteolytic activity when grown in submerged culture (Krayushkina et al. 1973; Poszar Hajnal et al. 1974). Cheese samples were prepared in the laboratory with 5 l of cows' milk and pilot scale studies with 100 l of milk using calf rennet, commercial substitutes and *M. miehei* rennet. Organoleptic evaluations conducted with ripened cheddar cheese made with these rennets indicated that cheddar cheese prepared with *M. miehei* rennet was quite comparable to that prepared with animal rennet and its market substitutes.

Effect of operational parameters

The effect of temperature on the milk clotting activity produced was studied at 30°, 40°, 42°, 45° and 50° C and the results are shown in Fig. 2. The organism used in this study is a thermophilic fungus and maximum enzyme activity was seen at 42° C. At higher and lower temperature ranges, there was a considerable decline in activity.

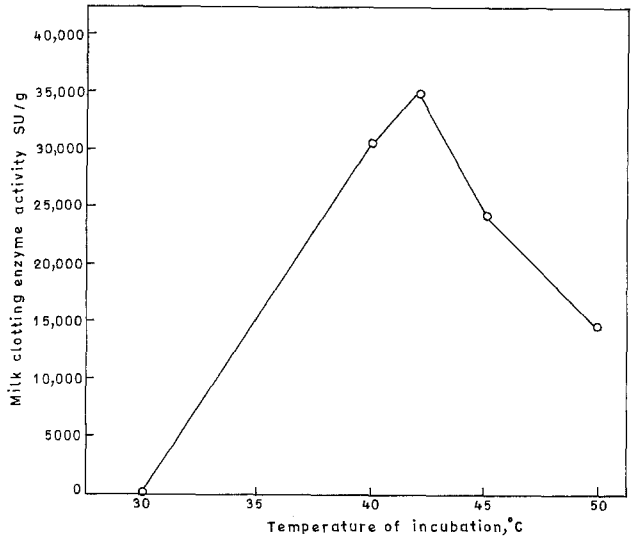

Fig. 2. Effect of temperature on milk clotting enzyme production (wheat bran to wheat flour ratio 3:1, incubation time 120 h)

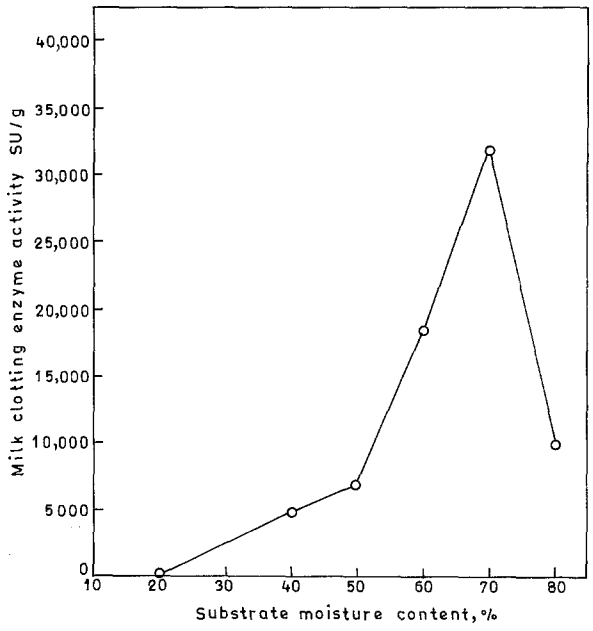

Fig. 3. Effect of substrate moisture level on milk clotting enzyme production

In another set of experiments the influence of the moisture content of the solid substrate medium on the protease activity was examined (see Fig. 3). Highest milk clotting activity was obtained at 70% moisture level and above and below this level a marked decrease was noticed.

Experiments on the effect of the initial pH of the mineral solution indicated that maximum protease activity (33456 SU/g) was obtained when the basal medium pH was 1.0. These optimum operational parameters were used for further studies.

Effect of nutritional parameters

Source of starch. Experiments were conducted using wheat-bran medium supplemented (30% based on total weight) with different sources of starch including the flours of rice, wheat and sorghum, tapioca and wheat starches as well as starch hydrolysate. Compared to the medium without starch supplementation which gave a yield of 19000 SU/g of milk clotting activity, various starch and flour supplements gave reasonably higher enzyme activities (23000–24500 SU/g). The starch hydrolysate however, was detrimental to enzyme activity, probably due to the catabolite repressive effect.

Of the different supplements tried above, wheat flour was found to be the best. From experiments varying the extent of this supplementation, a composition of wheat bran and wheat flour in the ratio 90:10 gave the highest yield (34000 SU/g) of milk clotting enzyme and this medium was used for further studies.

Nitrogen source. Experiments were carried out with different nitrogen sources, both inorganic and organic. Inorganic (sodium nitrate, urea and ammonium nitrate — media A, B, C) sources were added to give a nitrogen concentration of 0.5% and organic sources (skim milk powder, gelatine, defatted soybean and groundnut flours — media D, E, F, G) were added at a 1% (w/w) concentration. The results are shown in Fig. 4. Sodium nitrate and urea in the medium gave very poor yields of enzyme but ammonium nitrate supported good growth and moderate enzyme formation. Organic nitrogen sources favoured good enzyme yields, the maximum being 40287 SU/g with gelatine, followed by skim milk powder, defatted groundnut cake and soybean flour respectively. The control experiment with wheat bran and wheat flour in the ratio of 90:10 but no externally added nitrogen (media H in Fig. 4) gave a milk clotting enzyme activity of 34000 SU/g.

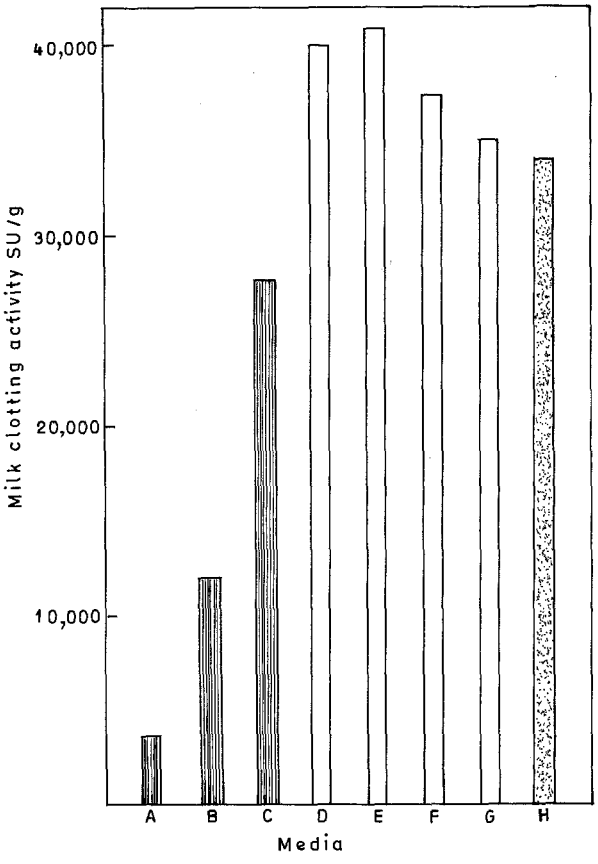

Fig. 4. Effect of nitrogen sources on the production of milk clotting enzyme. The cultivation medium contained wheat bran and wheat flour (90:10), 70% moisture content, temperature 42° C, time of cultivation 120 h. *A*, sodium nitrate; *B*, urea; *C*, ammonium nitrate; *D*, skim milk powder; *E*, gelatine; *F*, defatted soybean; *G*, groundnut flour; *H*, no added nitrogen

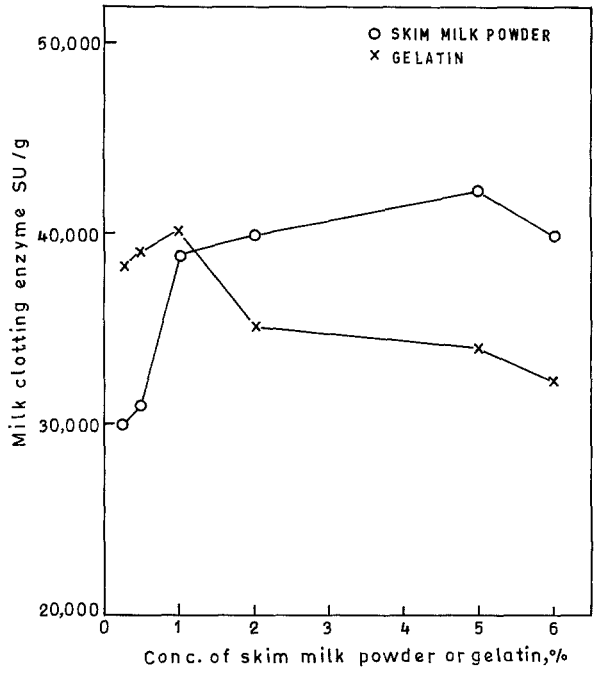

Fig. 5. Effect of concentration of skim milk powder (O---O) and gelatine (×---×) on the production of milk clotting enzyme. Fermentation parameters are as given in Fig. 4

On the basis of the above results, skim milk powder and gelatine were chosen as nitrogen sources for a study of the effect of their concentration (0.25%–5%) on enzyme production and the results are depicted in Fig. 5. Gelatine showed little effect on enzyme activity up to a concentration of 1%, after which it showed a decline. In the case of skim milk powder there was a fairly steep increase in activity as the concentration increased to 1%, after which the activity very gradually increased to reach a maximum of 42900 SU/g at a concentration of 5%. Based on these results and in view of the cost of the raw material, 1% skim milk powder appears to be the one of choice.

From the results discussed above, the optimum conditions giving maximum yield of milk clotting enzyme can be summarised as follows: wheat bran and wheat flour (90:10); skim milk powder (1%), pH 1.0 (initial pH of mineral solution); temperature 42° C; moisture content 70%.

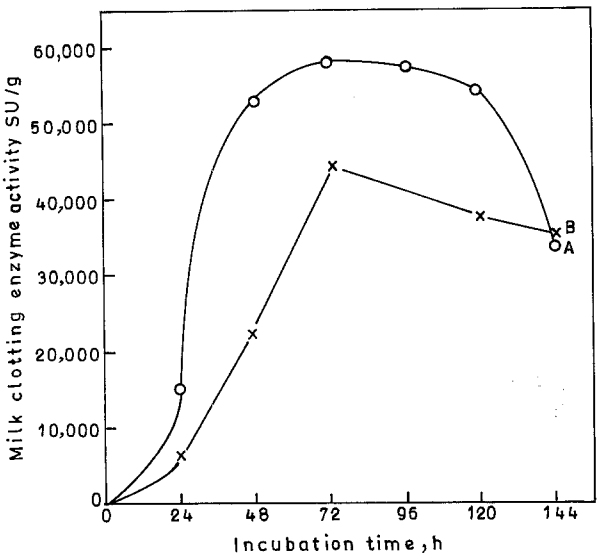

Fig. 6. Time course of enzyme production using optimised cultural and nutritional parameters in flasks (*A*, O---O) and large-scale production in a koji room (*B*, ×---×)

Small and large scale studies with optimized parameters

Selecting the optimum cultural and nutritional parameters determined earlier, experiments on a small scale at flask level were carried out and the time course data for a typical run is shown in Fig. 6. The milk clotting activity increased sharply during the period 24–48 h and then at a declining rate to reach a maximum (58000 SU/g) at 72 h. After remaining more or less constant until 96 h, it decreased gradually up to 120 h and then fell sharply.

Large-scale production of the enzyme was carried out in a koji room with the optimised parameters and the time course of enzyme activity obtained is also shown in Fig. 6. The activity profile with time was sharper than the flask experiment and maximum activity was obtained at 72 h fermentation time but the highest activity reached was lower at 45262 SU/g.

A comparison with the published results of other workers on the production of milk clotting enzyme under submerged fermentation and SSF can be made. Higashio and Yoshioka (1981, 1982) screened several fungi and found that *M. racemosus* under SSF could produce a milk clotting enzyme activity of 6500 SU/g but a low 600–800 SU/ml in liquid medium. Ismail et al. (1984) reported that milk clotting enzyme production was favoured by surface cultivation in comparison with submerged fermentation. Pozsar-Hajnal et al. (1974) reported that *M. pusillus* ladt. produced 20000–60000 SU/g in wheat bran medium under SSF condition.

The milk clotting enzyme activities obtained in the present experiments with *M. miehei* under SSF are promising from the point of view of the development of a commercial process. The yields obtained compare favourably with the highest milk clotting activity reported so far (Poszar-Hajnal et al. 1974) under SSF conditions. Further, a ratio of milk clotting to proteolytic activities of 3.5:1 has been achieved in the crude extract and 6.6:1 in the purified enzyme which is comparable to commercial rennet substitutes on the market.

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References

Arima K (1972) Conversion of foodstuffs by microorganisms, Proceedings of the 4th International Symposium of IU FOST Kyoto, Saikon Publishing Co. Ltd, Tokyo, Japan, p 99

Birkkjaer H, Johnk P (1985) Technological suitability of calf rennet substitutes. Bull Int Dairy Fed 194:8–13

Crewford RJM (1985) Future developments in rennet and its use in a cheese factory. Bull Int Dairy Fed 194:14–23

Higashio K, Yoshioka Y (1981) Studies on milk clotting enzymes from microorganisms. I. Screening test and identification of potent fungus for producing milk-clotting enzyme and improvement of its enzymic properties using mutants. J Agric Chem Soc Jpn 55:561–571

Higashio K, Yoshioka Y (1982) Milk clotting enzyme production by N.T.G. induced mutant of *Mucor racemosus* no. 50. J Agric Chem Soc Jpn 56:777–785

Ismail AMS, El-Aassar SA, Abdel Fattah AF (1984) Production of milk clotting and proteolytic enzyme by fungi. Agric Waste 10:95–102

Krayushkina EA, Zhebebtsov NA, Zvyagintsev VI (1973) Optimization of the composition of nutrient medium for cultivation of the mould *Mucor pusillus*, 917. Prikl Biokhim Mikrobiol 9:883–885

Krishnawamy MA, Nagaraja Rao KS, Sreekantiah KR, Mannar MC (1976) Production of fungal rennet substitute for cheese making. J Food Sci Technol 13:187–191

Kunitz M (1947) Crystalline soybean trypsin inhibitor. II. General properties. J Gen Physiol 30:291–310

Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951) Protein measurement with Folin-phenol reagent. J Biol Chem 193:265–275

Pozsar-Hajnal K, Vomos-Vigyazo L, Hegedus-volguesi E (1974) Investigation into the production of milk clotting enzyme preparation. I. Enzyme producing capacity of *Mucor pusillus* ladt. in surface culture. Acta Aliment Acad Sci Hung 3:73–82

Yuan DC, Ochi T (1986) Studies on microbial milk clotting enzymes produced by fungi in different media I. Jpn J Dairy Food Sci 35:A199–A204

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