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Fermentation parameter and partial biochemical characterisation of milk clotting enzyme from Chinese distiller's yeast

Jinju WU[1,2], Hongbing CHEN[1], Weiping CHEN[2*]

[1]State Key Lab of Food Science and Technology, Nanchang University, Nanchang 330047; [2]Jiangxi Agricultural University, Nanchang 330045, China

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Abstract -.A strain F34 producing milk clotting enzyme with high yield was obtained from Chinese distiller's yeast, using casein plate prescreening and fermentation reselection. The optimum fermentation medium for milk clotting enzyme production was as follows: 5.0% rice flour and bran digest, 5.0% bean flour digest, 0.5% $CaCl_2$, 0.5% KCl, initial pH 4.5. The optimal fermentation parameters was at 34 °C for 60 h. The enzyme action was optimal at 50 °C and at about pH 5.5. In the absence of substrate the enzyme showed good stability at 40 °C and pH 3.5. Ca^{2+}, Zn^{2+}, Fe^{3+} and Fe^{2+} showed stimulation on the milk clotting activity; K^{+} and Mg^{2+} showed weak stimulation; Na^{+}, Cu^{2+}, Co^{2+} and Li^{2+} showed inhibition. The results indicate that this milk clotting enzyme may partially substitute calf rennet in the cheese manufacture.

Key words: milk clotting enzyme, fermentation conditions, biochemical characterisation.

INTRODUCTION

Milk clotting enzyme is the primary active agent in the manufacture of cheeses. Milk clotting enzyme preparations are known to be always associated with proteolytic activities (PA). However, for the production of cheese it is necessary to use rennin possessing strong milk clotting activity (MCA) and the least PA to minimise dissolution of the curd. The worldwide increase in cheese production, along with the reduced supply of calf rennet, has led to an increase in the demand for alternative sources of milk coagulants (Cavalcanti *et al*., 2004). Many kinds of animals, plants and microbial proteases have been suggested as milk coagulants, and more attention has been focused on the production of milk clotting enzymes from microbial sources as rennin substitutes (Hashem *et al*., 2000). Among those bacterial and fungal rennin substitutes, those from *Mucor miehei* (Seker *et al*., 1998), *Penicillium oxalicum* (Hashem, 2000), *Myxococcus xanthus* (Petit and Guespin, 1992), *Bacillus mesentericus* (Stoeva and Mesrob, 1977), *Nocardiopsis sp.* (Cavalcanti *et al*., 2004), and *Mucor baciliformis* (Venera *et al*., 1997) are the most widely known.

There are massive microbial floras in Chinese distiller's yeast and many of them can produce milk clotting enzymes. The present work was undertaken to select milk clotting enzymes produced from the yeast and investigate the pattern of MCA and PA in the enzyme preparations under different culture conditions. Some properties of one of the selected milk clotting enzymes were also classified.

* Corresponding author. Phone: +86.0791.3813420;
Fax: +86.0791.8333708; E-mail: wujinju302@hotmail.com

MATERIALS AND METHODS

Microorganism. Four kinds of Chinese distiller's yeast were obtained from Jiangxi Agricultural University,China. Other six kinds of Chinese distiller's yeast were obtained from local markets.

Inoculum and culture medium. Casein plate was composed of 0.1% dextrose, 1.0% casein, 0.5% skim milk powder and 2.0% agar. The initial inoculum and fermentation medium was comprised of 5.0% rice flour and bran digest, 5.0% bean flour digest, initial pH 6.0. Each 250 ml Erlenmeyer flask contained 50 ml of the medium autoclaved at 121 °C for 15 min. Transfers were made from the stock cultures to potato dextrose slants medium, which were then incubated at 32 °C for 2 days. An inoculum culture was obtained by culturing the strains in the initial inoculum medium at 32 °C for 24 h with shaking at 160 rpm.

The main cultivation was carried out by using 10% of the inoculum in the above medium and incubating at different temperatures for different periods with shaking at 160 rpm. Thereafter the fermented medium was filtered to separate the mycelium from the culture filtrate. The experiments were conducted in triplicate and the results were the averages of these three independent trials.

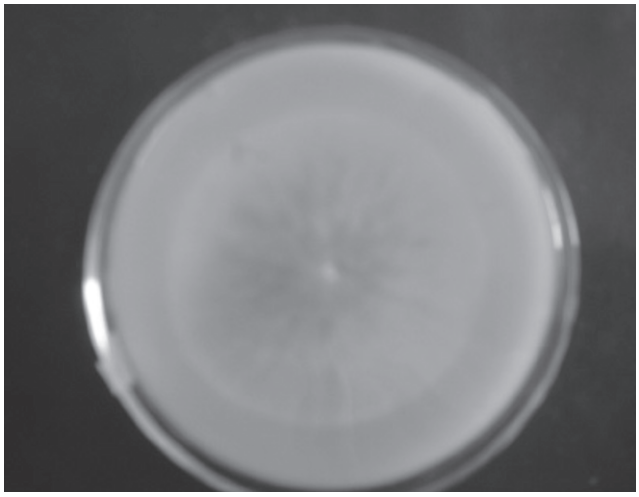

FIG. 1 - The colony characters of F34 in casein plate.

Effect of temperature on milk clotting enzyme activity and stability. For determination of optimum temperature of milk clotting activity, the reaction solution containing 10% skim milk (w/v in deionised water) was incubated over a temperature range of 30-60 °C. The thermal stability was determined by pre-incubating the enzyme in the temperature range of 40-60 °C. The incubation time of samples varied from 10 to 60 min. After incubation, the samples were submitted for determination of residual milk clotting activity.

Effect of pH on milk clotting enzyme activity and stability. To determine optimum pH of the enzyme, the reaction solution containing 10% skim milk (w/v in water deionised) was varied over the pH range of 5.5-7.2 using 0.1 N NaOH or HCl to adjust. To determine pH stability, the enzyme was adjusted over the pH range of 1.5-6.5 and preincubated for 24 h at 0 °C or 1 h at 30 °C. After incubation, the samples were submitted for determination of residual milk clotting activity.

Effect of metal ions on milk clotting activity. MCA was measured in the presence (final concentration 1, 3 or 5 mM) of various metal ions. The MCA of control was measured in the absence of metal ions and looked as 100%.

Assay of enzyme activity. MCA was measured by the method of Arima and Iwasabe (1967). One unit of milk clotting activity was defined as the activity which clotted 1 ml of 10% skim milk (w/v in water deionised) in 40 min. PA of the culture was estimated according to the method of Bergkvist (1963). One unit of PA was taken as the amount of enzyme which liberated 1 μg of tyrosine per ml per minute.

RESULTS AND DISCUSSION

Selection of strains producing high yield milk clotting enzyme

One hundred and four strains were isolated from ten kinds of Chinese distiller's yeast and then cultivated in casein plate at 30 °C. Forty seven strains could produce white precipitation area and ten of them with the highest precipitation were selected for further tests. Fig. 1 shows the colony characteristics of F34 in casein plate. The MCA of F34 strain reached 33.3 U/ml and the value of MCA/ PA was 11.9. Since both values were the highest within the ten strains, strain F34 was further studied.

The results of phenotypic characteristics indicated that the strain F34 belonged to *Phycomycetes, Mucorales, Mucoraceae, Rhizopus, Rhizopus arrhizus* Fischer.

Effect of different medium components and physico-chemical parameters on milk clotting enzyme production

Effect of various ratios of carbon sources

During the microbial fermentations, the carbon source acts not only as a major constituent for building of cellular material, but also as important energy source (Dunn, 1985).

Using rice flour and bran digest as composite carbon sources, the ratio of rice flour digest to bran digest was 5:0, 4:1, 3:2, 2:3, 1:4 and 0:5, respectively. The ratio had great effect on MCA. When the ratio was 2:3, MCA reached 33.52 U/ml and MCA/ PA was maximum (Table 1). Other ratios were found to be poor substrates with respect to both milk clotting enzyme production (from 10.89 to 23.71 U/ml) as well as the value of MCA/PA (from 2.43 to 5.61). Hashem (1999) found that sucrose (5%) was optimum for the production of milk clotting enzyme from *Penicillium oxalicum*.

Effect of various nitrogen sources

Bean power digest was the best nitrogen source. When using bean power digest as organic nitrogen source, MCA reached 37.47 U/ml and MCA/PA was the maximum (about 9.78). Peptone and yeast extract were unsuitable because they decreased MCA, i.e. 15.61 and 31.4 U/ml, respectively. Hashem (1999) found that the original mixture of yeast extract (0.3%) plus peptone (0.5%), or baker's yeast alone (on equivalent N-basis) were the best nitrogen sources, but corn steep and soybean were unsuitable because they increased caseinase activity but milk-clotting activity decreased.

Using bean power digest as organic nitrogen source, we determined the effect of various inorganic nitrogen sources, such as NH_4NO_3, $(NH_4)_2SO_4$, NH_4Cl, $NaNO_3$ and $(NH_4)_2HPO_4$, on MCA. The results indicated that inorganic nitrogen sources were detrimental to MCA, which ranged from 6 to 9 U/ml. We hypothesize that the organic nitrogen source can satisfy the need of enzyme production. This is consistent with the previous report that inorganic nitrogen sources gave poor production of MCE, but organic

TABLE 1 - Effect of composite carbon on the production of enzymes

Ratios of composite carbon	5:0	4:1	3:2	2:3	1:4	0:5
MCA (U/ml)	11.91	4.66	1.72	33.52	23.71	10.89
PA (U/ml)	3.631	3.45	4.15	3.70	4.23	4.49
MCA/PA	3.282	4.25	5.23	9.06	5.61	2.43

TABLE 2 - Effect of metallic salts on the production of enzymes

Metallic salts	MgSO4	FeSO4	CaCl2	ZnSO4	KCl	Control
MCA (U/ml)	22.55	2.04	52.3	9.66	50.05	44.39
PA (U/ml)	6.49	1.1	5.12	3.3	3.61	5.02
MCA/ PA	3.47	1.85	10.21	2.93	13.86	8.84

nitrogen sources (wheat bran, wheat four, skim milk powder and soybean flour) favoured good enzyme yield by *Mucor miehei* and *Streptomyces clavuligerus (*Thakur *et al*.,1990; Porto *et al*., 1996). Otherwise, adding inorganic nitrogen source may affect the ratio of carbon to nitrogen, which is disadvantageous to enzyme production.

Effect of various metallic salts and initial pH

Adding 0.5% metallic salts, such as $FeSO_4$, $CaCl_2$, $ZnSO_4$, KCl and $MgSO_4$, to basal medium, it resulted that $CaCl_2$ and KCl was stimulatory the milk clotting enzyme production, while $FeSO_4$ and $MgSO_4$ was inhibitory (Table 2). When adding $CaCl_2$ and KCl to basal medium, MCA was 52.30 and 50.05 U/ml, respectively, whereas the value of MCA/PA was higher (10.21 and 13.86, respectively) than the control (8.84). Abdel Fattah *et al*. (1984) reported that K^+ had an adverse effect on the milk clotting/proteolytic activity ratio, while addition of Mg^{2+} to the culture medium did not significantly affect production of the milk-clotting enzyme.

Unlike other initial parameters, the pH of 4.5 was found to be optimum for MCA. At initial pH 4.5, MCA was the highest (61.58 U/ml). An initial pH value of the culture medium of 5.0 gave the highest milk clotting/proteolytic activity ratio and hence good rennin properties of the enzyme from *Absidia cylindrospora* (Abdel Fattah *et al*., 1984). While maximum MCA production by *Mucor miehei* (Escobar and Barnett, 1993) and *Bacillus subtilis* natto (Shieh *et al.*, 2008) was reported to be in the culture with an initial pH of 6.5 and 6.0, respectively. Shieh *et al.* (2008) indicated that the optimal initial pH of the medium for MCE production may vary depending on the culture medium and the test organism.

Effect of fermentation temperature and time

Figures 2 and 3 show the effect of different fermentation temperatures and times on MCA and MCA/PA. When the culture was fermented at 32°C for 60 h, MCA was the highest, i.e. 165.79 U/ml, and MCA/PA reached to 24.30. While MCA was 161.14 U/ml, and MCA/PA was the highest (27.88) when the culture was fermented at 34 °C for 60 h. A temperature of 34 °C was more optimal for proteinase production than milk clotting enzyme production, whereas 28 and 30 °C were not suitable for both the enzyme activities. Overall, 34 °C for 60 h resulted the best conditions to fit with these two conditions. Several researchers reported (Thakur, 1990; Hashem, 1999; Tubesha, 2003) that the optimum fermentation periods for the production of milk clotting enzyme were between 3-8 days; it was different from one strain to another.

Preliminary biochemical characterisation of milk clotting enzyme of strain F34

Effect of temperature on milk clotting activity and stability

To study the influence of temperature on the milk clotting activity, a temperature range from 30 to 60 °C was selected. The enzyme activity was maximum at 50 °C and the enzyme still possessed good activity at 45 °C (74.6% of that at 50 °C). These results are in disagreement with those reported by Masanobu (1970) who found that MCA from *Basidiomycetes* was optimum at 55 to 60 °C. MCA from *Penicillium oxalicum* and *Rhizopus oryzae* was also optimum at 60 °C (Hashem, 2000; Kumar, 2004). Cavalcanti *et al.* (2004) indicated the MCA from *Nocardiopsis* sp. was highest at 55 °C. The milk-clotting enzyme produced by *Aspergillus versicolor* was optimal at 45 °C (Abdel Fattah et al., 1988). Calf rennet is optimum at 50 °C (Chao, 2004), and it's in agreement with our result.

After heating the enzyme solution before incubation with skim milk at 40 °C for 60 min it still possessed 100% of its original activity (Fig. 4). At 45 °C, the enzyme showed good stability and retained 90% activity after 30 min. But above 50 °C, it

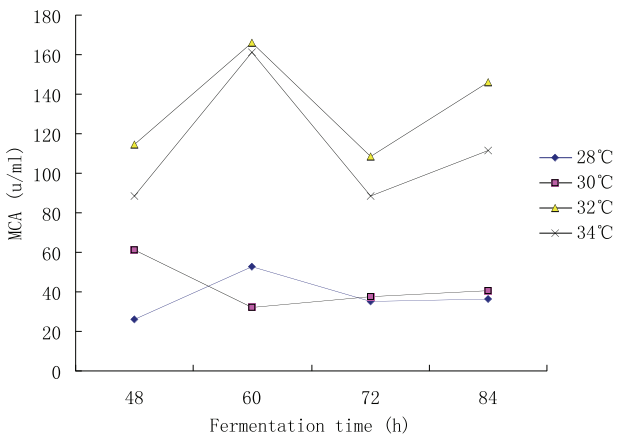

FIG. 2 - Effect of fermentation temperature and time on the production of milk clotting enzyme.

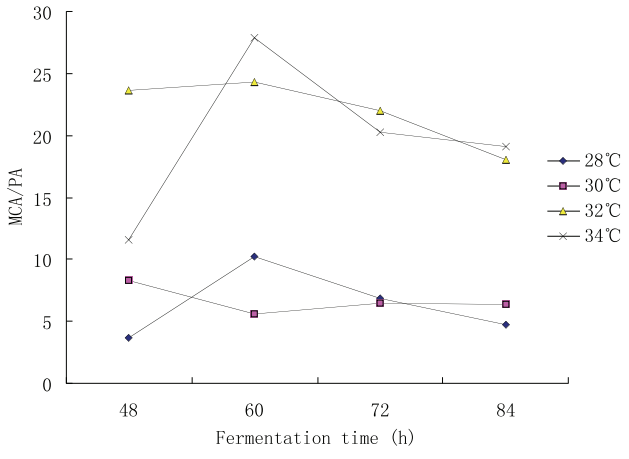

FIG. 3 - Effect of fermentation temperature and time on the milk clotting activity/proteolytic activities (MCA/PA).

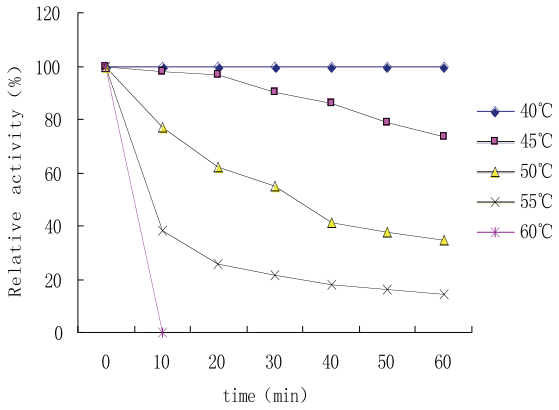

FIG. 4 - Effect of temperature on enzyme stability of milk clotting enzyme.

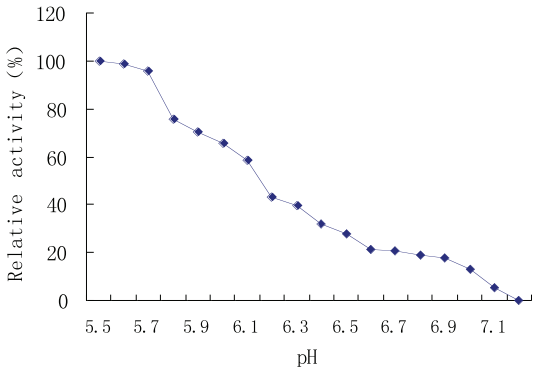

FIG. 5 - Effect of milk's pH on enzyme activity of milk clotting enzyme. To determine optimum pH of the enzyme, 10% skim milk (w/v in water deionised) was varied over the pH range of 5.5-7.2 using 0.1 N NaOH or HCl to adjust. Milk clotting enzyme was added to the solution to measure the relative activity.

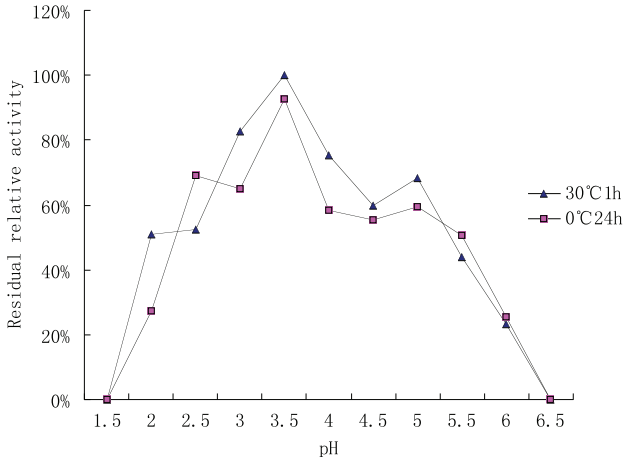

FIG. 6 - Effect of pH on enzyme stability of milk clotting enzyme. The enzyme was adjusted over the pH range of 1.5-6.5 and preincubated for 24 h at 0 °C or 1 h at 30 °C. After incubation, the samples were submitted for determination of residual milk clotting activity.

caused greatly loss in activity. As soon as the temperature was up to 60 °C, MCA was completely lost. MCA from *Penicillium oxalicum* has the similar characteristics: the enzyme showed good stability at 40 °C, and the enzyme retained 52% activity after 30 min at 50 °C, but 60 °C caused dramatic loss in activity (Hashem, 1999). Calf rennet was also stable up to 45 °C and lost its activity at 60 °C (Chao, 2004). The result from this report indicated that the enzyme maybe a good substitute to calf rennet.

Effect of pH on milk clotting activity and stability

The enzyme showed a maximum activity at pH 5.5. An increase in the pH of skim milk was accompanied with a gradual loss of MCA. When the pH was above 7.0, almost all of MCA lost (Fig. 5). The result is in accordance with Venera *et al*. (1997), who observed an optimum pH of 5.5 for MCA from *Mucor baciliformis*. Hashem (2000) also observed that an increase in the pH of the reaction mixture was associated with a gradual loss of MCA, but at pH 7, 86.79% of the enzyme activity of *Penicillium oxalicum* was lost and it exhibited a maximum activity at pH 4. All of the commercial milk clotting enzymes showed the same trend: when pH decreases from 7 to 5, MCA increases quickly (Hashem, 1999). However, Cavalcanti *et al.* (2004) reported that optimum activity of crude and partially purified extract produced by *Nocardiopsis* sp. was displayed at pH 11.0 and pH 7.5, respectively. The milk clotting enzyme produced by *Aspergillus versicolor* was optimal at pH 6 (Abdel Fattah et al., 1988).

The enzyme showed good stability at about pH 3.5 (Fig. 6). When incubation ranged from pH 2.0 to 5.0 for 1 h at 30 °C or pH 2.5 to 5.5 for 24 h at 0 °C, MCA retained more than 50% of the original activity. When pH was below 1.5 or above 6.5, whether kept for 1 h at 30 °C or 24 h at 0 °C, MCA was completely lost. Calf rennet was stable at pH 3 to 5. Masanobu (1970) reported that milk clotting enzyme from *Basidiomycetes* kept stability at pH 3 to 4, which was in agreement with our research. Buffalo chymosin was stable within pH interval 2.0-6.0, but was almost complete inactivated near pH 7.0 (Abdel Malak *et al.*, 1996). The fact that milk clotting enzyme produced in this study showed a wide range of pH stability has added advantage to its usefulness as cheese-making coagulant. Therefore, based on these characteristics, we conclude that the milk clotting enzyme from Chinese distiller's yeast might be a suitable rennet substitute for the production of cheese.

Effect of metal ions on milk clotting activity

Figure 7 showed that Ca^{2+}, Zn^{2+}, Fe^{3+} and Fe^{2+} had great stimulating effects on MCA. Comparatively, Fe^{3+} and Fe^{2+} were the most effective, and in their presence at 5 mM concentration the activity reached 1.81- and 2.02-fold higher than that in their absence. Ca^{2+} had the optimum effect at 3 mM concentration (1.62-fold of the control), compared with Zn^{2+} at 5 mM concentration (1.36-fold of the control). K^{+}、Mg^{2+} had slight stimulating effects (1.08-, 1.17-, 1.08-and 1.04-, 1.02-, 1.06-fold of that in their absence) at 1, 3 and 5 mM concentration, respectively. Na^{+}, Cu^{2+}, Co^{2+} and Li^{2+} showed inhibition on milk clotting activity. Li^{2+} showed the strongest inhibition on milk clotting enzyme at 5 mM concentration, about 67% of the control. Chao (2004) reported that Ca^{2+} and Fe^{2+} had great stimulating effects on calf rennet, while Cu^{2+} inhibited the activity. Hg^{2+}, Ag^{+}, Zn^{2+}, and K^{+} reduced the MCA, whereas Cu^{2+} and Mn^{2+} activated the MCA from *Mucorpusillus* var. *Lindt* (Arima *et al.*, 1968). Cu^{2+}, Co^{2+}, and Mg^{2+} had stimulating effects on the enzyme from *Penicillium oxalicum*, while Na^{+}, EDTA effected inhibition (Hashem, 2000).

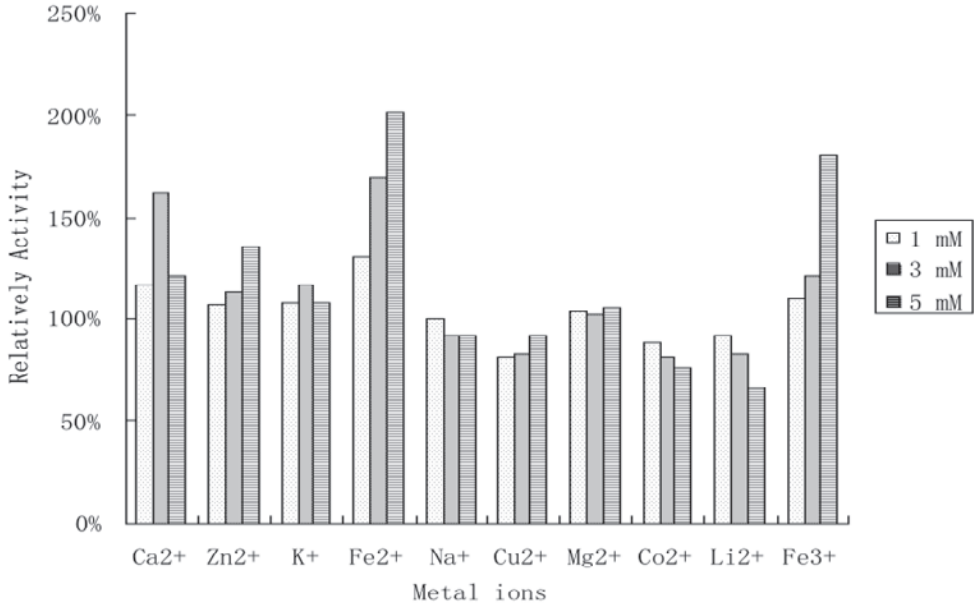

FIG. 7 - Effect of metal ions on enzyme activity of milk clotting enzyme.

CONCLUSIONS

Numerous attempts have been made to replace calf rennet because of limited supply and increasing high prices (Chazarra *et al*., 2007). A strain F34 producing milk clotting enzyme with high yield was obtained from Chinese distiller's yeast. When using the optimum fermentation parameters, MCA was 161.14 U/ml, and MCA/PA was the highest (27.88). The enzyme possessed strong MCA and weak PA, and the enzymological characteristics were similar to calf rennet. The results obtained in this study on the fermentation parameter and partial enzymological characteristics of milk clotting activity indicate the possibility of the use of this milk clotting enzyme in the cheese manufacture.

Acknowledgments

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