CHAPTER V

CHARACTERIZATION OF MILK CLOTTING ENZYME PRODUCED BY *P. acidilactici* SH AND *L. paracasei* CF1

5.1 INTRODUCTION

Milk clotting activity is greatly influenced by temperature, pH and calcium chloride concentration that influence the enzymatic coagulation of milk as well as rheological properties of the formed gel. Milk clotting activity is strongly influenced by the temperature and affects protein aggregation (Najera et al., 2003). It was reported that increased temperature increases the rate of gel firming and causes the protein matrix to shrink due to increased hydrophobic interaction (McMahon et al., 1984). Additionally, temperatures higher than normal milk pasteurization temperature causes extensive degradation of casein leading to bitter taste, reducing the yield of the gel and totally denature the enzyme (Lucey, 2002). At higher incubation up 60°C significantly decrease the milk clotting activity (Foda et al., 2012). A decrease in the pH of milk to below 5.8 leads to a change in the distribution of casein between micelles and serum (Awad, 2007).

Several microbial extracellular proteinases show activity similar to rennin and are suitable for cheese-making, and the activity varies with bacteria, pH and temperature. It was reported *B. subtilis natto* (Shieh et al., 2009) showed maximum enzyme activity at pH 6 and temperature 37°C; *B. badius* MTCC 7727 exhibits optimum activity at pH 50 and 40 °C (Rao et al., 2007); a partially purified neutral protease from *B. subtilis* showed optimum MCA at pH 7.0 and 40°C (El-Safey et al., 2004) a halotolerant *B.*

aquimaris VITP4 produced extracellular protease with optimum pH 7.5 and temperature 37°C (Shivanand et al., 2009). A thermophillic neutral protease from *Bacillus* strain HS 08t showed optimum MCA at pH 7.5 and 65°C. The milk clotting enzyme obtained from *B. subtilis* YB-3 showed maximum activity at 70°C with pH stability ranging from 5.0 to 9.0. This enzyme exhibited high specificity to β-casein of yak milk casein (Li et al., 2012). While *Streptomyces pseudogriseolus* NRC-15 showed maximum enzyme activity at pH 6.5 and temperature 45°C (Mostafa et al., 2013).

Any milk clotting enzyme both from microbial and plant sources should have specificity on κ-casein similar to renin. The optimum activity of animal renin at pH 6.3 and temperature 45°C and this enzyme is active with high specificity for cleaving at Phe105-Met106 peptide bond of k-casein. Rennet substitute from microorganisms usually showed MCE with high MCA with high MCA/PA ratio (Walstra et al., 1999). Sato et al. (2004) reported that the LAB identified as *E. faecalis* TUA 2495 L have ability to produce MCE with milk clotting activity, and highest MCA was recorded at pH 6.5 and 70°C with high specificity on k-casein. Stoeva and Mesrob (1977) reported that MCE produce by *B. mesentericus* 76 showed maximum MCA at pH 7.5 and temperature at 60°C.

Calcium chloride play has a significant role on casein aggregation during milk coagulation (Rao & Mathur, 1979). Increasing Ca^{2+} concentration decreased MCA due to the increase of ionic force or to the saturation of negative residues of the micelles at increasing Ca^{2+} concentration in the medium (Merheb-Dini et al., 2010).

Therefore, in this study the effect of temperature, pH and calcium chloride concentration on the MCA, and the proteolytic activity of MCE produced by two LAB (*P. acidilactici* SH and *L. paracasei* CF1) were evaluated.

5.2 MATERIALS and METHODS

5.2.1 Effects of pH on Milk Clotting Activity and Proteolytic Activity

The optimum pH of MCA was determined at 50°C for *P. acidilactici* SH and 40°C for *L. paracasei* CF1 at different pH values using the following buffers: 0.2 M acetate buffer (p H 5.5 to 6.5); phosphate buffer pH (7 to 7.5) and Tris HCL (pH 8). Skim milk (10% w/v) was dissolved in the buffer and adjusted to the set pH with 0.1 M NaOH or 0.1 M HCl. For each pH, a control was carried out without the enzyme. MCA and PA were determined as described in Chapter 3.3.2. The PA was determined at 30°C and at pH values ranging from pH 5.5 to 8.

5.2.2 Effects of Temperature on Milk Clotting Activity and Proteolytic Activity

The optimum temperature for MCA and PA was determined by incubating the reaction mixture at different temperatures ranging from 25 to 60°C, with 5°C intervals at pH 6 and 7. MCA and PA were determined as described in Chapter 3 3.2.

5.2.3 Effect of Calcium Chloride Concentration on Milk Clotting Activity

Milk substrates supplemented with different concentrations of $CaCl_2$ were prepared at various concentrations (0.005; 0.01; 0.015; 0.02; 0.025; 0.03; 0.035 M). MCA and PA were determined as described in Chapter 3 3.2.

5.2.4 Urea SDS-PAGE analysis of Milk Clotting Enzyme on Various Milk and Casein Fractions

The action of the MCE on casein fractions was evaluated by 10% of urea SDS-PAGE at variable concentrations (0.2 to 2 mg/mL) of commercial bovine αs-, β- and k-casein (Sigma-Aldrich Quı´mica S.A.) dissolved in 100 mM sodium phosphate buffer pH 6.2, and 0.5 ml enzyme was added. The mixtures were incubated for 10 min at room temperature. After that, aliquots were taken and the reaction stopped by adding equal volumes of sample loading buffer (125 mm Tris-HCl, pH 6.8, 20% glycerol, 1% SDS, 0.01% bromophenol blue and 1.5 ml 2- β-mercaptoethanol) and heated at 95°C for 5 min. The sample was analyzed by urea SDS-PAGE. Electrophoresis was performed on a vertical gel apparatus (Mini-Protean, Bio-Rad Laboratories S. A.) with 10% precast Tris-Gly gels (Bio-Rad Laboratories S.A.) as described by Laemmli (1970). Proteins were stained with Coomassie brilliant blue.

5.2.5 Statistical Analyses

A one way analysis of variance (ANOVA) using MINITAB version 16 was carried out and the P value was set 0.05 ($P<0.05$) for significant differences. Tukey's Pairwise Comparison was used to compare mean differences on the effects of temperature and pH and their interactions on milk clotting activity.

5.3 RESULTS

5.3.1 Effect of pH on Milk Clotting Activity and Proteolytic Activity

A change of pH from 6.0 to 8.0 at incubation temperature of 50ºC significantly ($p<0.05$) affected MCA and MCA/PA for the MCE produced by *P. acidilactici* SH. The results obtained showed that at pH 6.0, maximum MCA of 75 SU/ml and MCA/PA of 37.5 was recorded. Increasing pH at 0.5 unit increment from 6.5, 7, 7.5, 8 and 8.5 decreased MCA values to 58, 54, 43, 21 and 17 SU/ml, and MCA/PA values to 30, 19, 12, 14 and 13, respectively (Figure 17).

The PA was evaluated at different pH ranging from 5.5 to 8.5 at an incubation temperature of 50ºC for 30 min (Figure 18). There was a gradual increase in PA value as the pH increased. The highest PA value was recorded at pH 7.5 with a value of 3.6 U/ml and MCA/PA of 12. However, the PA value decreased at pH 8.5 to 1.3 U/ml (Figure 19).

The MCA and MCA/PA value of enzyme produced from *L. paracasei* CF1 was highest at pH 7 with values of 57 SU/ml and 21, respectively. However, increasing the pH to 7.5, 8 and 8.5 at incubation temperature of 40ºC for 30 min decreased MCA values to 55, 43 and 30 SU/ml, respectively. The PA recorded the highest value at pH 7.5 (3.4 U/ml) and MCA/PA (16.2) (Figure 20). A decrease in PA was observed as pH increased to 8.0 (2.2 U/ml) and 8.5 (1.8 U/ml). These results clearly indicate that the enzyme is a neutral enzyme.

5.3.2 Effects of Temperature on Milk Clotting Activity and Proteolytic Activity

The MCA and MCA/ PA of both *P. acidilactici* SH and *L. paracasei* CF1 were assayed at different temperatures ranging from 25 to 60°C at constant pH of 6.0 and 7.0, respectively. The temperature significantly ($p<0.05$) affected the values of MCA *P. acidilactici* SH ; the MCA and PA for the enzyme produced were active between 30 to 55°C, but the activity was destroyed at 60ºC (Figure 21). Enzymes produced by this isolate showed highest MCA and MCA/PA value of 75 SU/ml 37.5 respectively at 50ºC, the MCA value reduced to 46 SU/ml at 55ºC and lost activity at 60ºC. Similarly, the PA value was affected significantly ($p<0.05$) by the temperature of incubation. The highest PA value was 3.4 U/ml at 45ºC, respectively (Figure 22).

Temperature also significantly ($p<0.05$) affected the MCE activity of *L. paracasei* CF1. The enzyme recorded the highest MCA and MCA/PA at a peak temperature of 40ºC. The MCA, and MCA/PA were 57 SU/ml, and 16.2 respectively (Figure 23). On the other hand the highest PA was 3.5 U/ml with reduce the ratio of MCA/PA to19 at 45°C (Figure 24)

Figure 17: Effect of pH on MCA by *P. acidilactici* SH Enzyme at 50°C

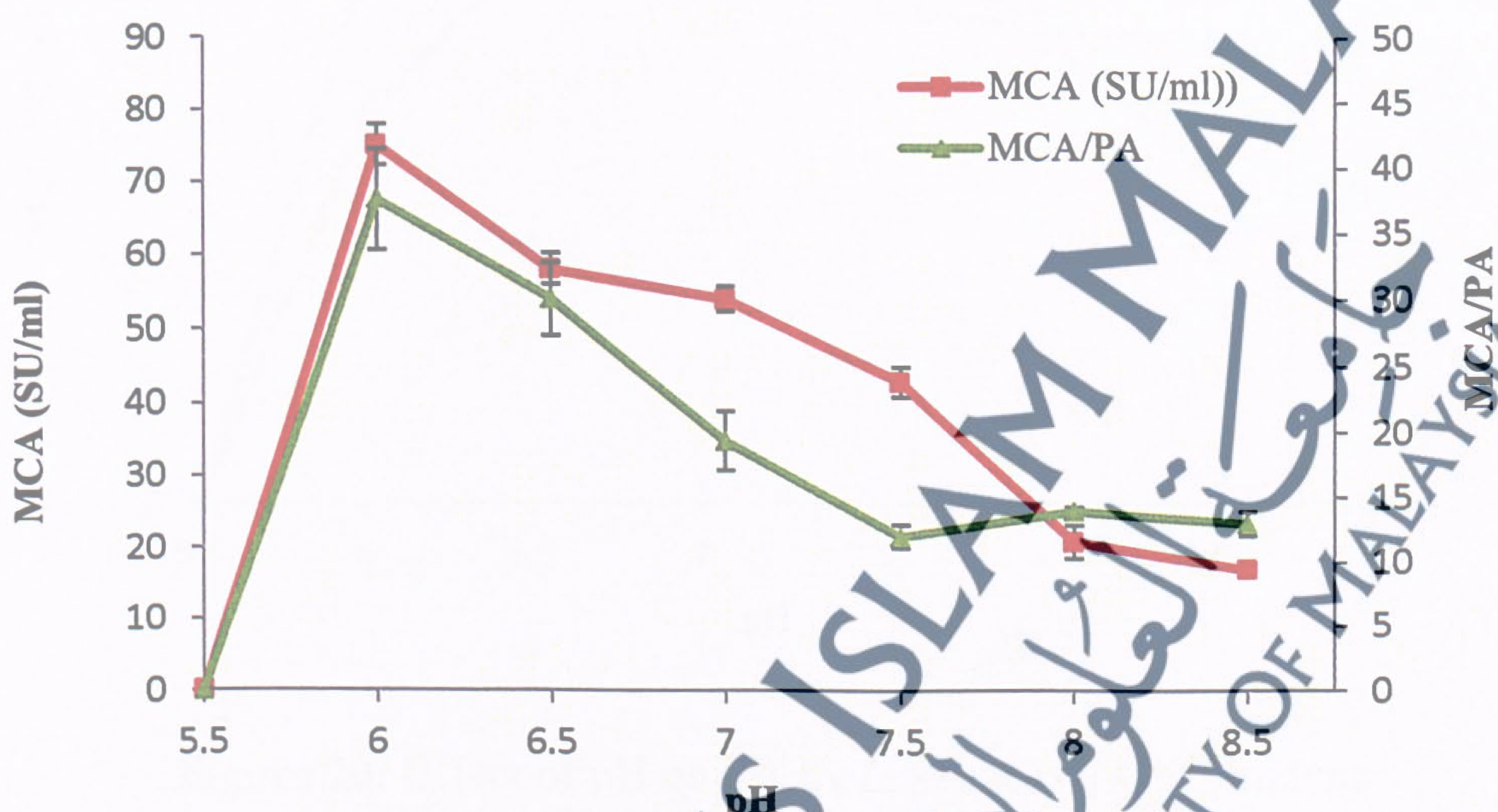


Figure 18: Effect of pH on PA by *P. acidilactici* SH Enzyme

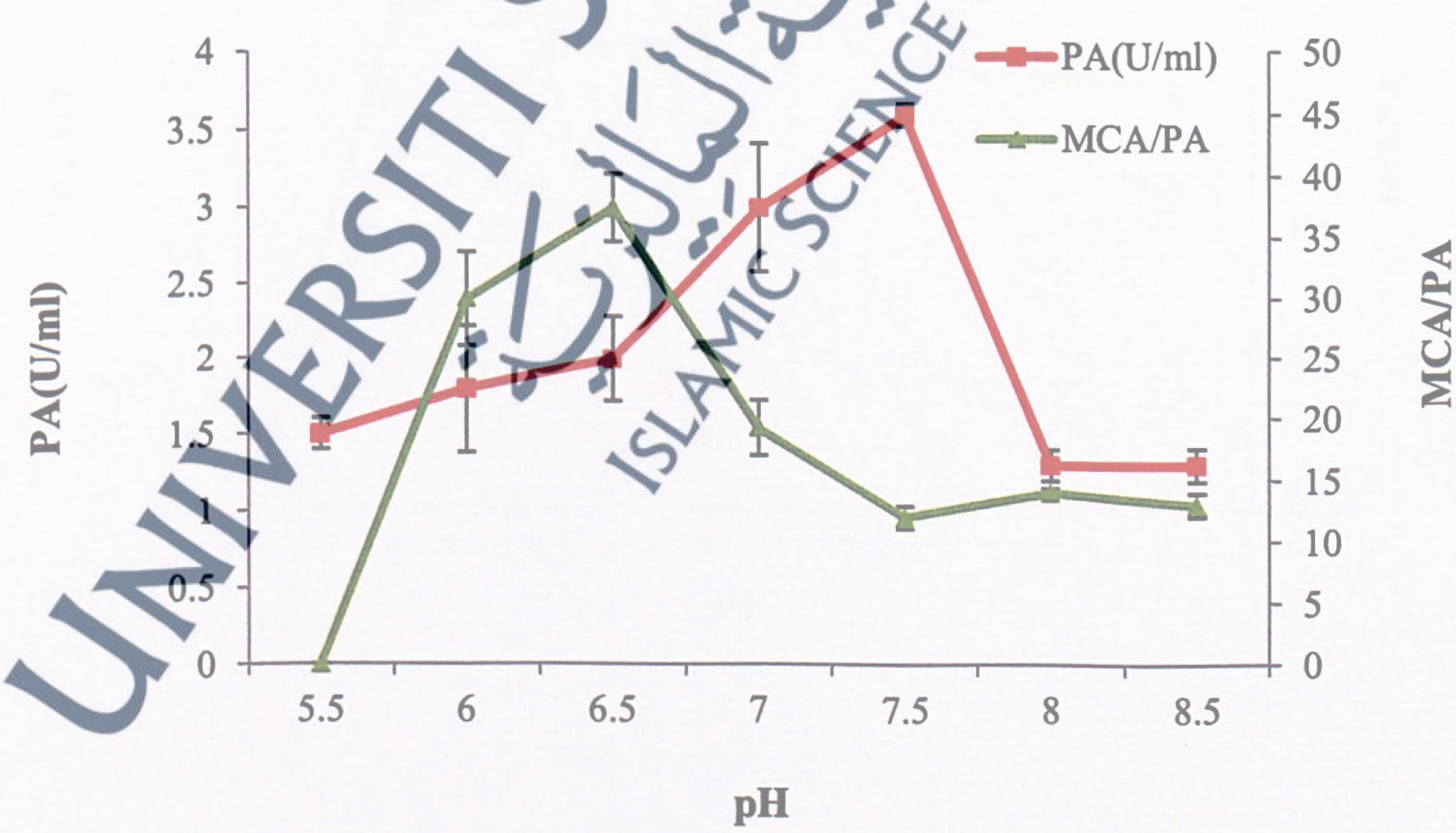


Figure 19: Effect of pH on MCA by *L .paracasei* CF1 Enzyme

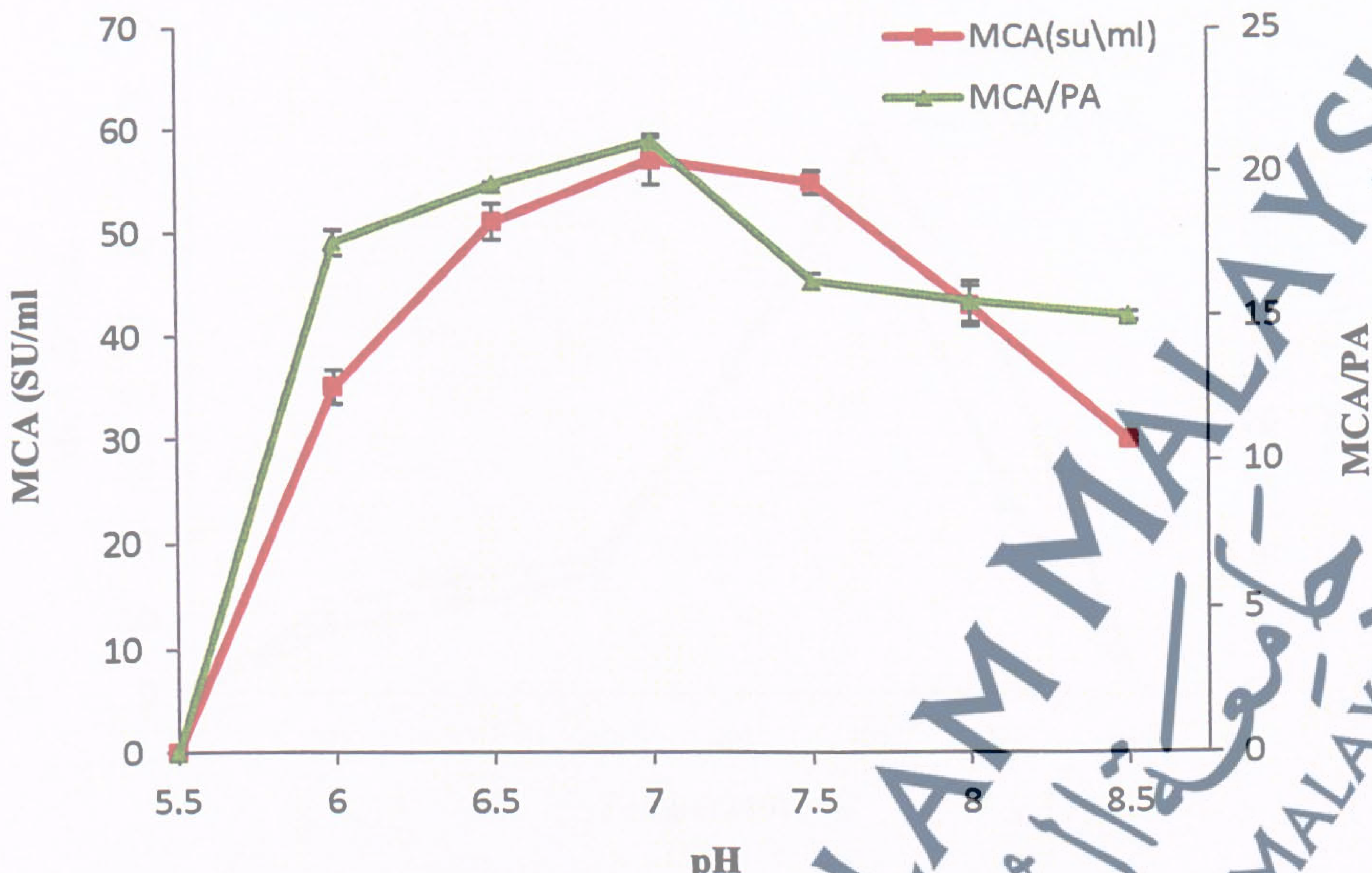


Figure 20: Effect of pH on PA by *L. paracasei* CF1 Enzyme

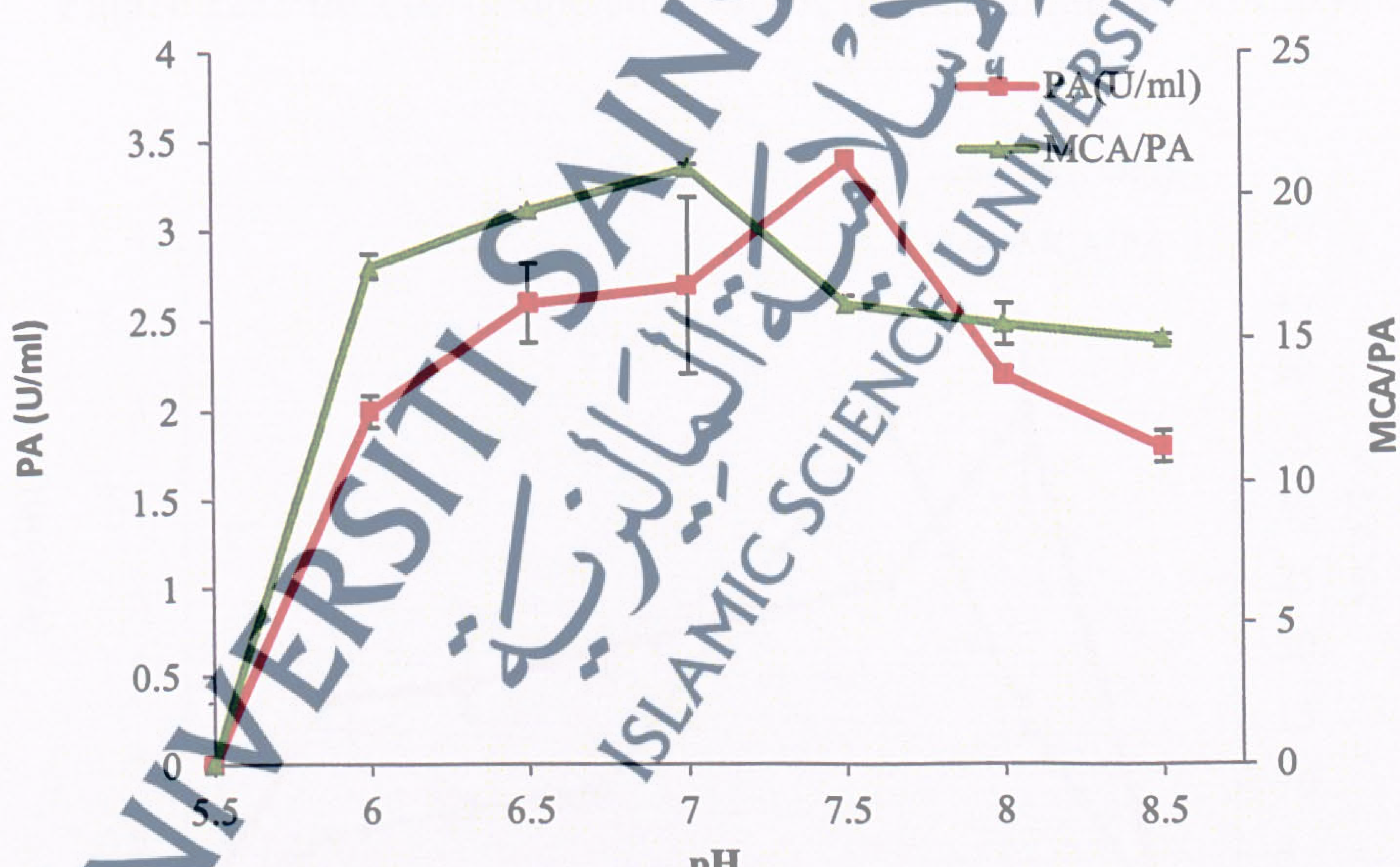


Figure 21: Effect of Temperature on MCA by *P.acidilactici* SH Enzyme

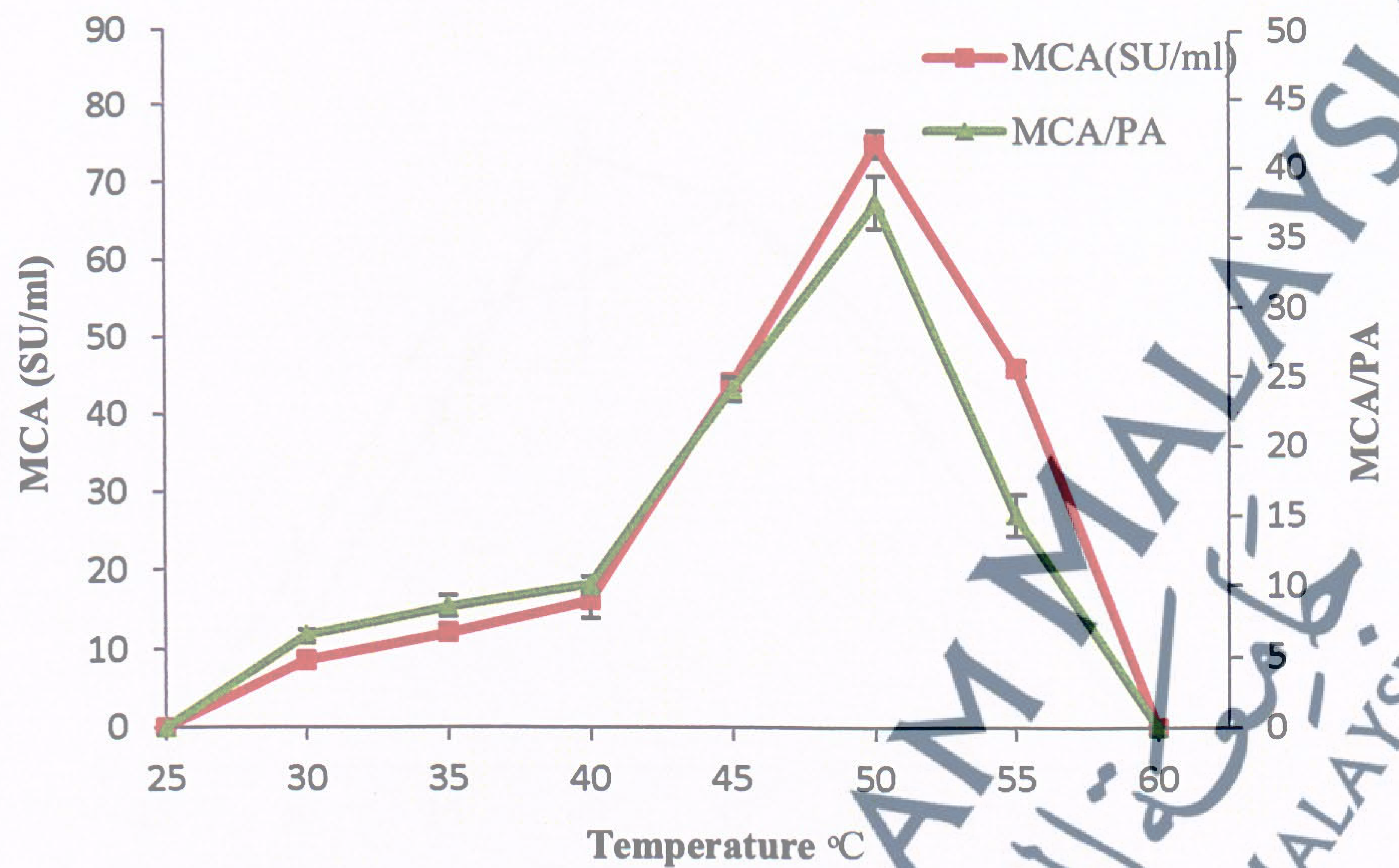


Figure 22: Effect of Temperature on PA by *P. acidilactici* SH Enzyme

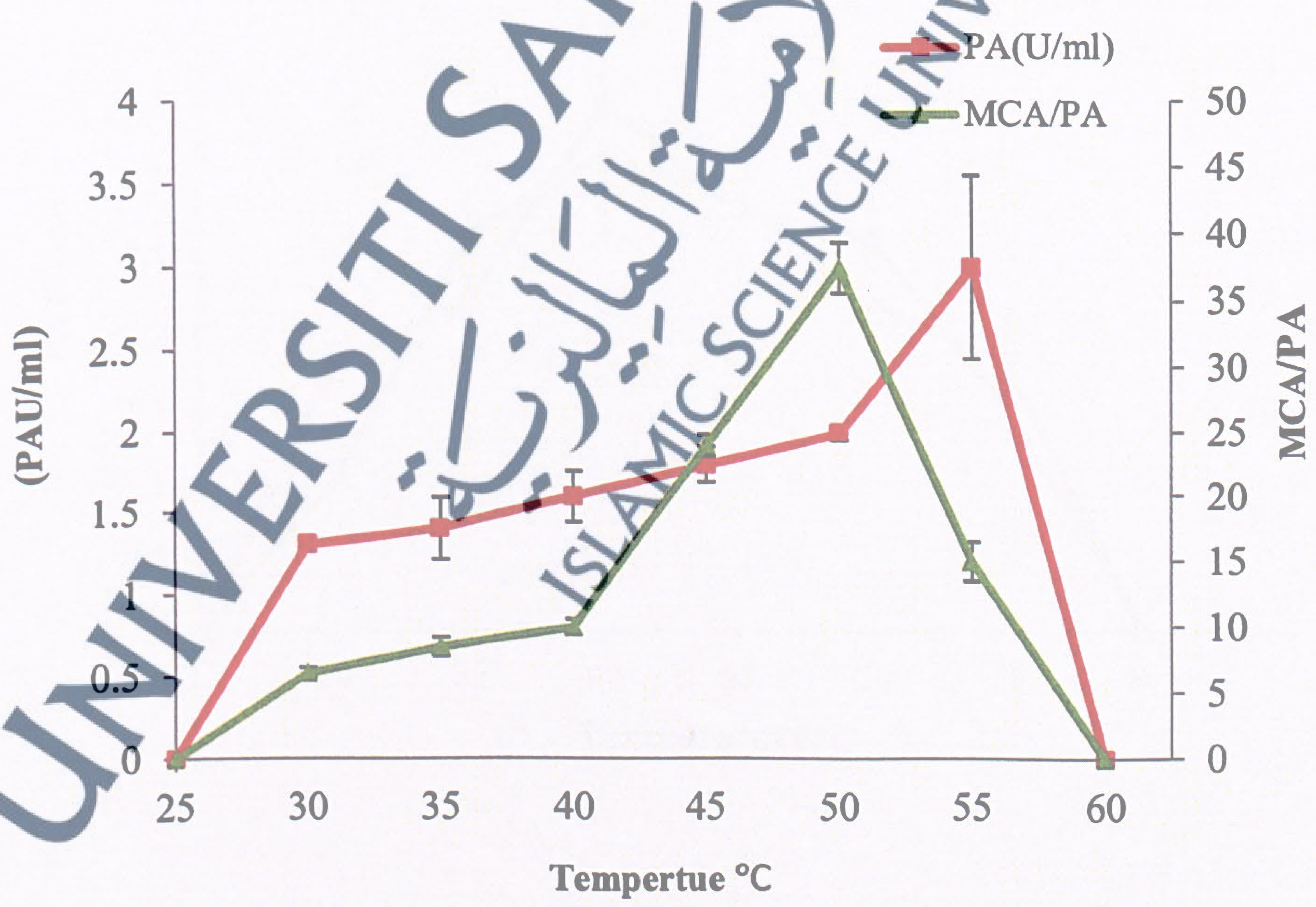


Figure 23: Effect of Temperature on MCA by *L. paracasei* CF1 Enzyme

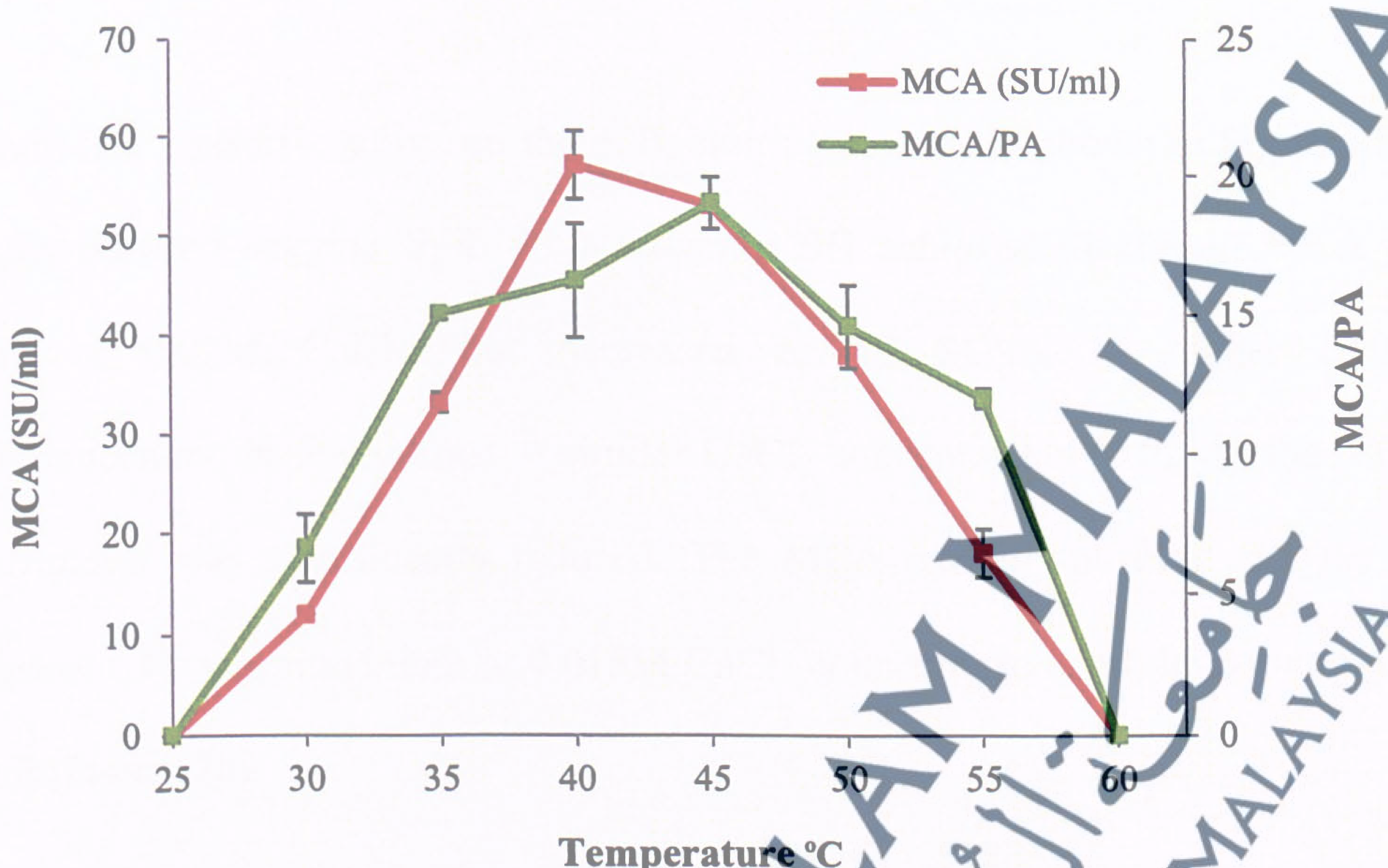
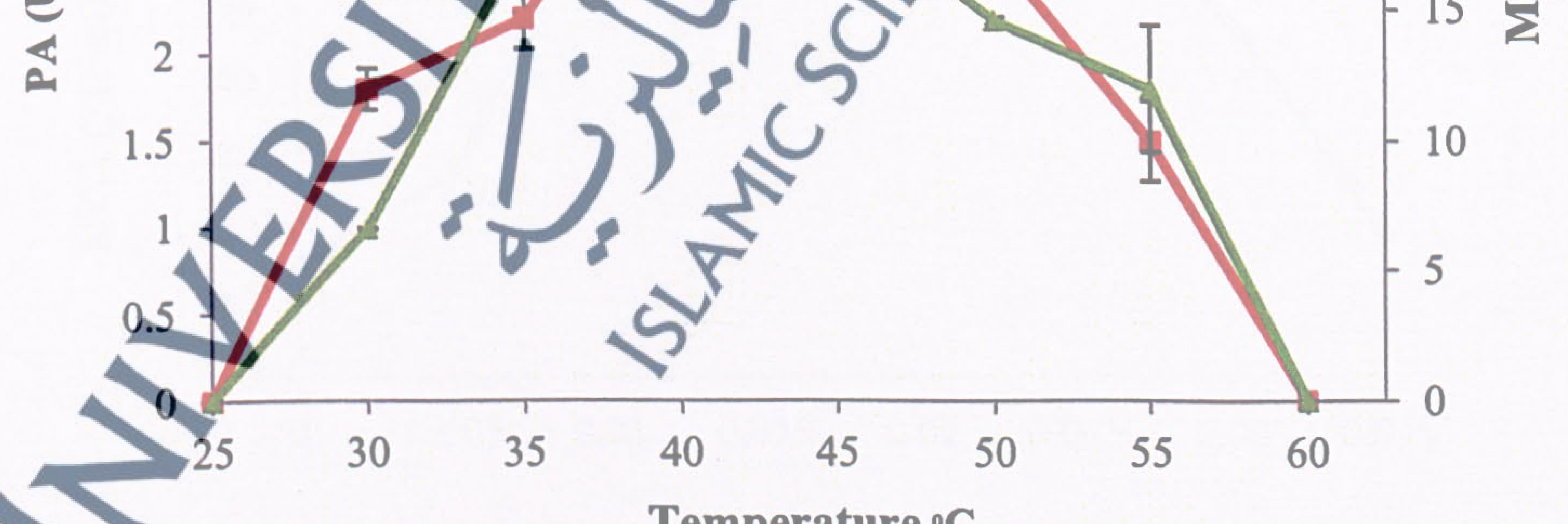


Figure 24: Effect of Temperature on PA by *L. paracasei* CF1 Enzyme



5.4.3 Effect of Calcium Chloride Concentration on Milk Clotting Activity

Calcium had a positive effect on the milk clotting activity as shown in Figure 25. The partially-purified enzyme from *P. acidilactici* SH achieved maximum MCA of 77 SU/ml at 0.02M $CaCl_2$, but decreased to 19 SU\ml at higher (0.035M) $CaCl_2$ concentration. In contrast at similar $CaCl_2$ concentration (0.02M), the MCA of *L. paracasei* was significantly reduced. The MCA partially-purified enzyme of *L. paracasei* CF1 was maximum at 0.015M $CaCl_2$ concentration and the activity was 55 SU/ml (Figure 26).

Figure 25: Effect of $CaCl_2$ Concentration on MCA of Enzyme Produced by *P.acidilactici* SH

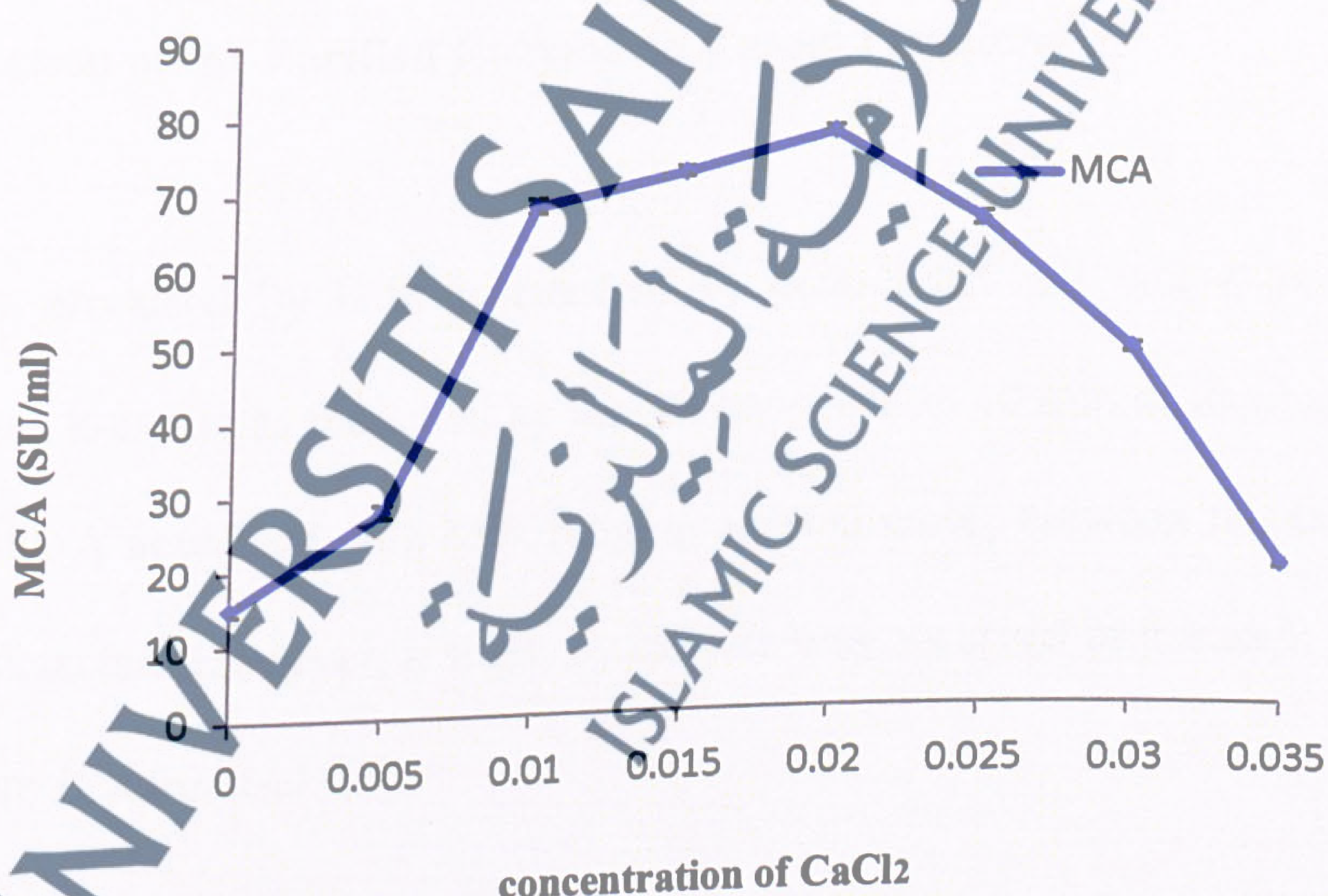
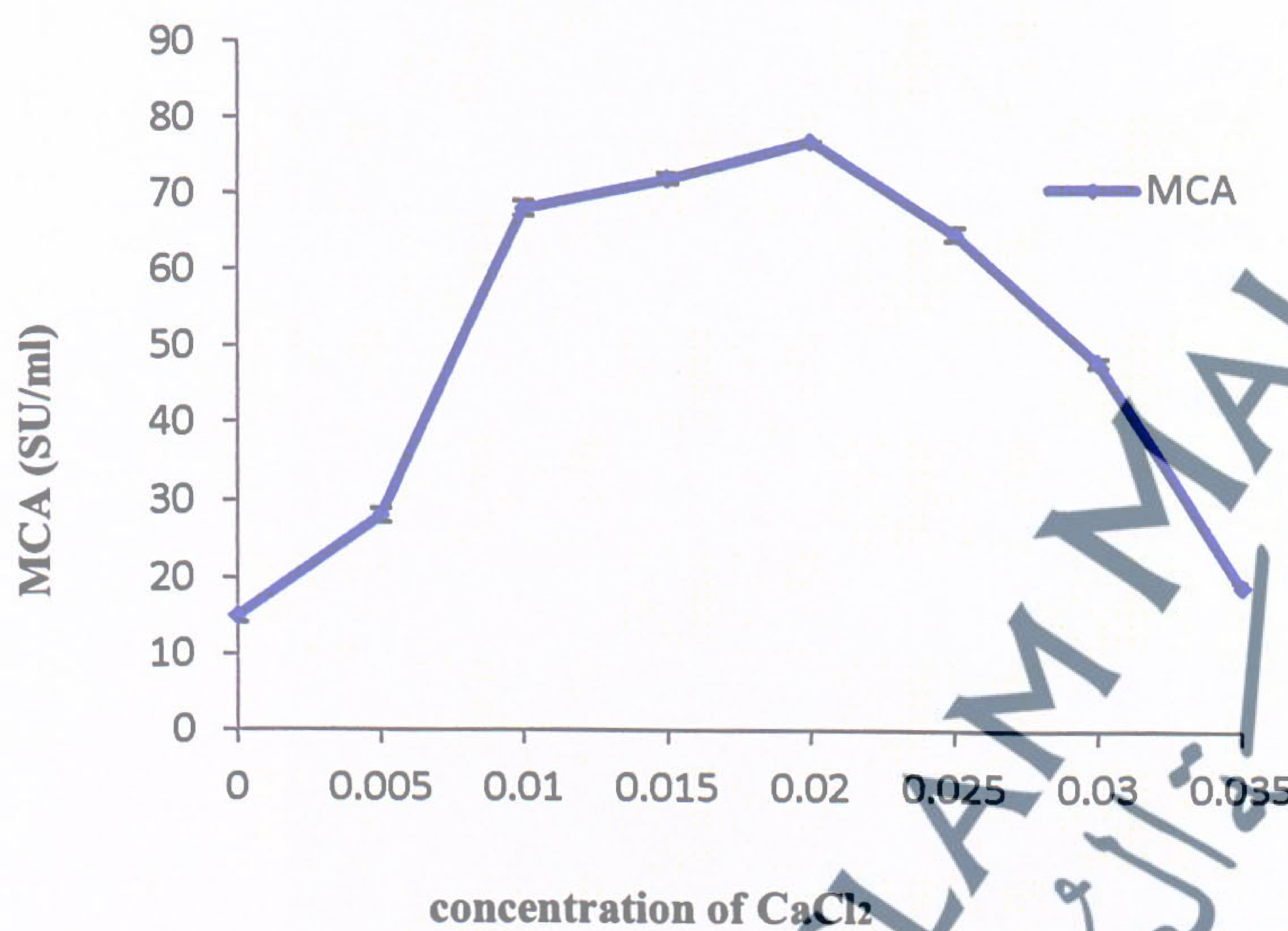


Figure 26: Effect of $CaCl_2$ Concentration on MCA of Enzyme Produced by *L. paracasei* CF1



5.3.4 Action of the Purified Enzyme on Casein Fractions

Enzymes produced by both isolates of *P. acidilactici* SH and *L paracasei* CF1 hydrolyzed k-casein as indicated by loss of band within 10 min of incubation (Figures 27 and 28). A new band with MW ranging approximately between 10,000 and 11,000 Da was detected. However, a slight hydrolysis was observed in α-casein produced by MCE from *L. paracasei* CF1.

Figure 27: Urea- PAGE of Casein Hydrolysis by *P. acidilactici* SH Enzyme. Lane1 α- CN. Lane 2 β- CNlane 3 whole CN. Lane 4 k- casein. Lane 5 Contains Molecular Weight Protein Markers

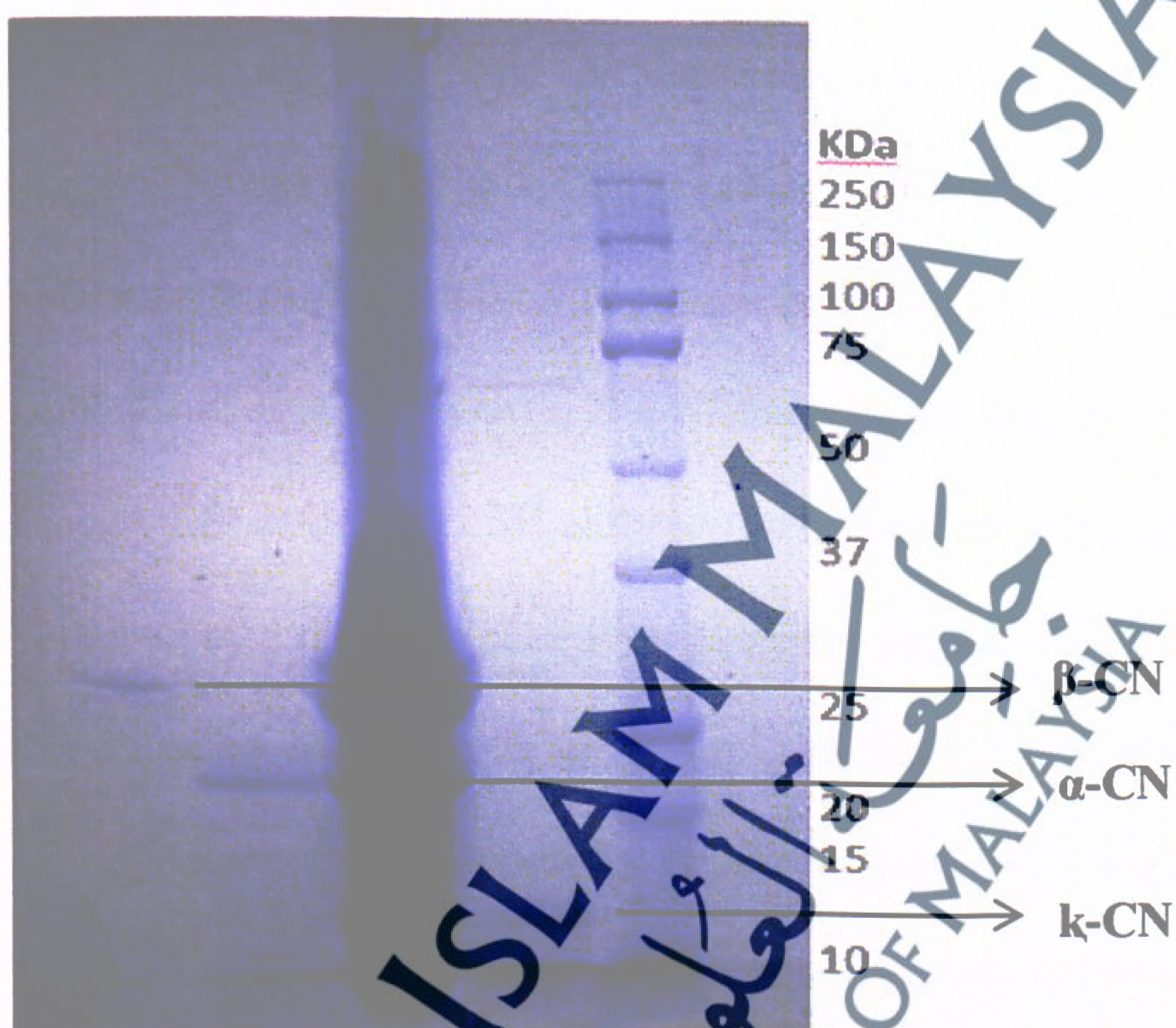
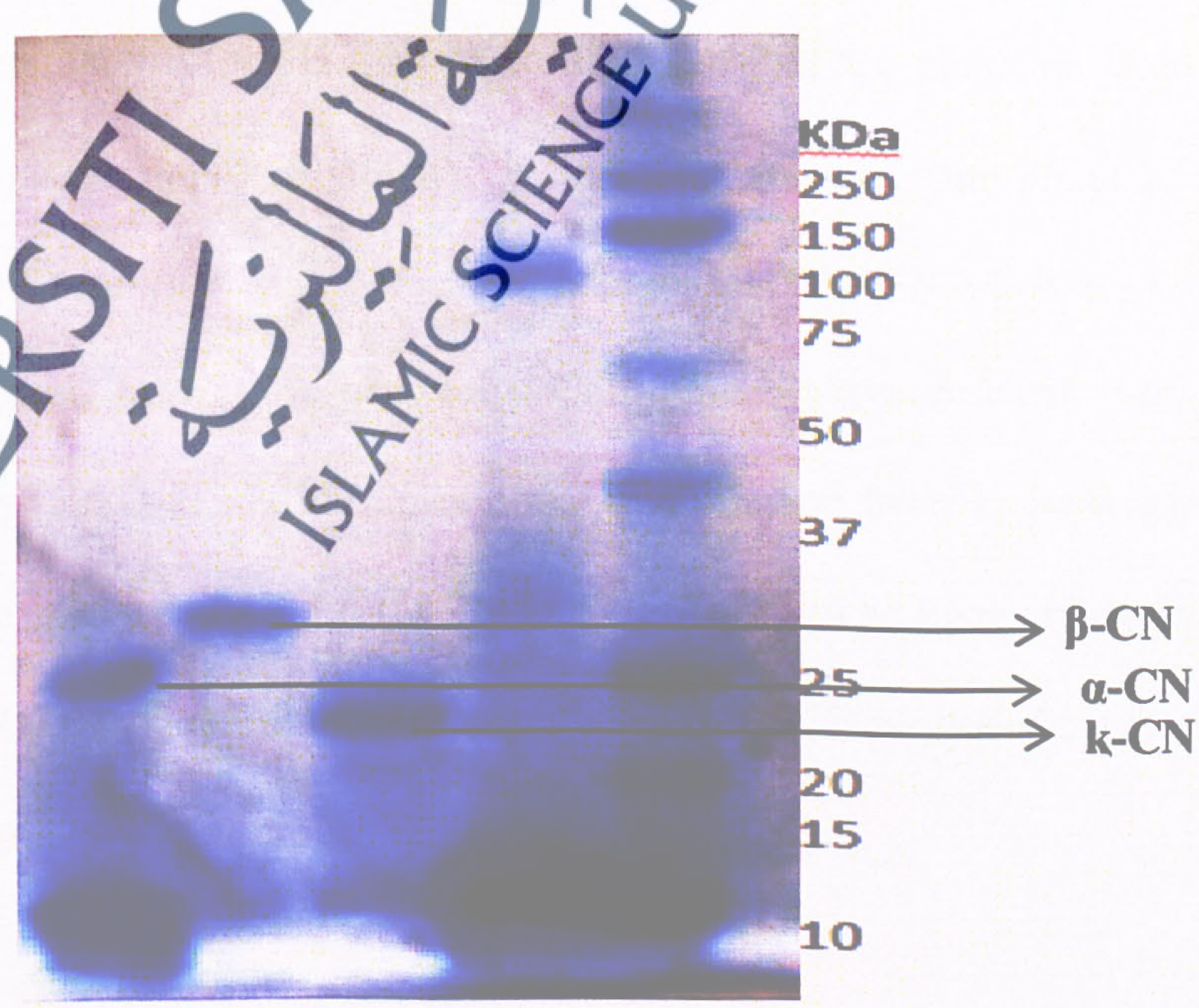


Figure 28: Urea- PAGE of Casein Hydrolysis by the *L. paracasei* CF1 Enzyme. Lane1 α- CN. Lane 2 β- CN Lane 3 k- CN. Lane 4 whole CN lane 5 Contains Molecular Weight Protein Markers



5.4 DISCUSSION

Milk clotting activities of microorganisms have different characteristics and values. The activity of the MCE is affected by various factors, such as pH, temperature and calcium chloride. Generally, milk clotting activity could not be achieved below pH 5.5 because the casein coagulates at low pH even in the absence of the enzyme. In this study the enzyme produced by *P. acidilactici* SH showed the highest activity at pH 6.0 with MCA 75 SU/ml followed by a decrease in activity at higher pH values (> pH 6.0) indicating the enzyme is a neutral enzyme, similar to Wu et al. (2013) who observed that the optimum MCA of the enzyme produced by *B. subtilis natto* was at pH 6.0. The MCE from *E. faecalis* TUA2495L studied by Sato et al. (2004) exhibited the optimum milk clotting activity at pH 5.8 to 6.7.

PA of *P. acidilactici* SH increased from pH 5.5 reaching a maximum reaction rate at pH 7.5 then lost its activity at pH 8.5. In contrast, proteolytic enzymes from fungi generally exhibit maximum proteolytic activity at acidic pH (optimum pH at 5.5) such as protease from *T. aurantiacus* and *M. pusillus* as observed by Merheb et al. (2007) and Richardson et al. (1967), respectively. Calf rennet showed weak activity in alkaline conditions (He et al., 2011). In contrast, the enzyme from *L. paracasei* CF1 showed the highest activity at pH 7.0 (MCA 55 SU/ml) followed by decrease in MCA at higher pH (> pH 7.0) values, similar to as reported for MCE produced by *B. subtilis* FP-133 with highest activity at pH 7.0 (El-Safey et al., 2004).

Temperature have variable effect on MCA. In this study the highest MCA (75 SU/ml) was exhibited by *P. acidilactici* SH at 50°C and no activity at 60°C. However, the optimal milk-clotting enzyme activity by *B. subtilis* natto was at 60°C; but heating at 60°C for 60 min or 70°C for 5min, the milk-clotting activity was lost (Wu et al., 2013). *B. subtilis* natto and *B. cereus* dramatically lost their activity at 55°C (Melachouris & Tuchey, 1987; Shieh et al., 2009). The MCE of *B. licheniformis* was completely inactivated after 3 min at 70°C (D'souza & Pereira, 1979). Similar observation was reported for MCE from *A. oryzae* MTCC 5341 that was active at 55°C and no activity at 62°C (Vishwanatha et al., 2010). Sato et al. (2004) reported that MCA of the enzyme from *E. faecalis* TUA2495L was optimum at 70°C MCE. This result showed that, the MCE from *P. acidilactici* SH was destroyed at pasteurization temperature (63ºC). The MCE of *L. paracasei* CF1showed highest activity at 40ºC Rao et al. (2007) reported that *Bacillus badius* MTCC 7727 exhibited optimum MCA at 40ºC temperature.

Calcium chloride plays an important role in milk coagulation as well as in the gel formation. Increasing the concentration of $CaCl_2$ over 10mM may have a negative effect on curd formation, due to the additional calcium will increase the positive charge on the surface of the micelle causing charge repulsion and produces weaker gel or no gelation at all (Sandra et al., 2012). In this work the MCA of enzyme produced by *P. acidilactici* SH increased from 28 SU/ml to 77 SU/ml when $CaCl_2$ was included at 0.005M to 0.02M; MCA decreased at higher concentration of $CaCl_2$. Similarly, highest MCA activity of microbial rennet produced by *R. miehei* and *R. pusillus* was at 20mM $CaCl_2$ (Nouani et al., 2009). Increasing $CaCl_2$ concentration to 20mM in goat or cow milk decreased milk clotting time while at higher concentration of $CaCl_2$ prevents milk clotting activity. In contrast the enzyme from *L. paracasei* CF1showed highest MCA at 0.015M of $CaCl_2$ concentration. Vairo-Cavalli et al. (2005) suggested that the increase in Ca^{2+} concentration in the substrate increase the ionic force or the saturation of negative residues of the casein micelles preventing their aggregation to form the clot.

Milk clotting activity of the *P. acidilactici* SH enzyme completely hydrolysed k-casein but slightly hydrolyzed α-casein but not β casein as shown by urea.SDS-PAGE (Figure 10). Similar observation was reported by Sato et al. (2004) of which the enzyme produced by *E. faecalis* hydrolyzed k-casein but not α-casein and β- casein. Chymosin was reported to act on k-casein but not on α-caseins and β-caseins (Irigoyen, 2001). However, extracted enzyme from *T. aurantiacus* hydrolysed β-casein even after 60 min of incubation as reported by Merheb et al. (2007); the enzyme was used for the production of bioactive peptides, but not curd. This selective hydrolysis of casein fractions is very important for cheese making to produce high yield curd. The results of this study show that *P. acidilactici* SH has the enzyme properties that are ideal for the production of MCE similar to commercial rennet that can be used for some dairy products in gel formation.

5.5 CONCLUSION

The result obtained from this study suggests that both enzymes produced by *P. acidilactici* SH and *L. paracasei* CF1 are neutral enzyme. However, the enzyme obtained from *P. acidilactici* SH can be considered as a themophilic enzyme because the optimum. MCA temperature was at 50ºC while the enzyme produced by *L. paracasei* CF1 is mesophilic enzyme which recorded the highest MCA at 40ºC. Calcium chloride also showed to significantly influence the MCA at varying concentration in the enzyme activity of both isolates. The specific activity of the enzyme from *P. acidilactici* SH was on casein fractions that completely degraded k-casein, but *L. paracasei* CF1 degraded k-casein and slightly α-casein.