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Milk clotting and proteolytic activity of enzyme preparation from *Pediococcus acidilactici* SH for dairy products

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Some microorganisms have the ability to produce enzymes that could clot milk and used as a substitute for calf rennet. Strains of lactic acid bacteria (LAB) could produce proteolytic enzymes that may have the potential to be used as a source of milk clotting enzyme (MCE). In the present study, LAB isolated from shrimp paste identified as *Pediococcus acidilactici* SH, was evaluated for milk clotting activity (MCA), proteolytic activity (PA) and enzyme production using different sources of nitrogen. Casein, tryptophan, trypticase peptone and tryptone soya were added to the enzyme production media, and casein was found to increase the MCA to 75.0 SU ml^{-1} at pH 6.0 at 50°C. SDS-PAGE of the partially purified enzyme solution produced a band with a molecular weight of approximately 29 kDa. This study indicates the possibility of exploiting LAB from food sources for the production of MCEs for dairy production.

Keywords: Lactic acid bacteria (LAB), milk clotting activity, nitrogen source, *Pediococcus acidilactici.*

INTRODUCTION

Milk coagulation is a basic step in cheese manufacturing. For long time calf rennet, the milk clotting enzyme (MCE) extracted from the calf's fourth stomach, is the most widely used coagulant in cheese making all over the world to manufacture a variety of cheeses. Other sources of MCEs have been used commercially such as from *Rhizomucor miehei* (Merheb et al., 2010) while others are being considered as potential sources that include *Aspergillus oryza* (Shata, 2005), *Amylomyces rouxii* (Yu and Chou, 2005), *Thermomucor indicae-seudaticae* (Reps et al., 2006) and *A. niger* (Moosavi-Nasab et al., 2010). Attempts to study the production of MCE from bacteria have concentrated on *Bacillus subtilis* natto(Shieh et al., 2009), *B. sphaericus* (El-Bendary et al., 2007), *Streptomycetes* sp. (Kathiresan and Manivannan, 2007) and *Streptomyces clavuligerus* (Keila et al., 2001).

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Abbreviations: MCE, Milk clotting enzyme; **LAB,** lactic acid bacteria; **PA,** proteolytic activity.

Microbial enzymes are especially preferred since they can be mass cultured and offer a variety of properties that can be selected for the production of different types of cheese.

The MCE from plants such as *Ananas comosus, Carica papaya* and *Lactuca sativa* (Egito et al., 2007), *Bromelia hieronymi* (Mariela et al., 2010), *Solanum dubium* (Isma et al., 2009) and artichoke flowers *Cynara scolymus* L., (Chazarra et al., 2010) and fruits of plants *Balanites aegyptiaca, Albizia lebbeck* and *Helianthus annus* (Egito et al., 2007) have received attention especially, when the use of animal rennet might be limited for religious reasons, vegetarianism diet or consumer concern regarding genetically engineered foods as in Germany, Netherlands and France (Egito et al., 2007). Cheeses made with vegetable coagulants are normally produced on an artisanal scale, in farmhouse or small dairy. Unfortunately, most of them proved unsuitable for cheese making owing to their excess in proteolytic character which lowers cheese yield and produce bitter flavors in the final cheese (Roseiro et al., 2003).

In the production of cheese, lactic acid bacteria (LAB) are normally added as starter cultures to acidify the milk while the addition of rennet allowed coagulation of milk casein. Additionally, LABs are involved in flavor development during cheese ripening. Production of MCE from (LAB) for use as rennet substitutes would be an interesting consideration because LABs are known to produce proteolytic enzymes and generally recognized as safe (GRAS) status. While many investigations on LAB focused on the degradation of milk proteins in flavor development, limited work has been reported on the potential of using LAB as a source of MCE except those reported by Elvira et al. (2001) on *Lactobacillus helveticus* and Sato et al. (2004) on *Enterococcus faecalis* TUA2495L. Therefore, the purpose of this study was to evaluate MCA and PA of enzyme preparation from *Pediococcus acidilactici* isolated from food sources for possible application in dairy products.

MATERIALS AND METHODS

Isolation and screening of lactic acid bacteria

Lactic acid bacteria that produces extracellular proteinase using skim milk agar was sub-cultured in MRS agar incubated at 37°C for 24 h, anaerobically. Strains which formed good clot of casein with clear and little whey was selected for further study (Sato et al., 2004). Thus, *P. acidilactici* SH isolated from shrimp paste (belacan) was selected and identified using API CH50 kit and 16s rDNA was cultured on MRS agar (de Man-Rogosa- Sharpe) incubated at 37°C for 48 h, anaerobically (Aween et al., 2012).

Preparation of production media for MCE

The enzyme production media, consisted of trypticase peptone(10 g), glucose (10 g), $CH_3COONa•3H_2O$ (2 g), $MgSO_4•7H_2O$ (200 mg), $MnSO•4H_2O$ (10 mg), $FeSO_4•7H_2O$ (10 mg), NaCl (10 mg), dissolved in D.W. 1000 ml and pH was adjusted to 6.8. One millilitre of the 24 h LAB pre-cultured broth was inoculated into an Erlenmeyer flask containing 100 ml of the enzyme production medium without or with 1% $CaCO_3$ sterilized by autoclaved at 121°C for 15 min. The enzyme production media, with $CaCO_3$ was incubated in a shaker incubator (150 rpm) at 30°C for 48 h, followed by centrifuged at 9,000 x *g* for 20 min at 4°C. The supernatant was collected and filtrated by filter size 0.22 µ regenerated cellulose membrane filter for MCA and PA measurement, and MCA/ PA ratio (Sato et al., 2004).

MCE assay

Enzyme assay was carried following the method described by Arima et al. (1968) and Sato et al. (2004). The MCA was expressed as Soxhlet units (SU) in which 1 SU is defined as the amount of enzyme that clots 1 ml of a solution containing 0.1 g skim milk powder and 0.00111 g $CaCl_2$ in 40 min at 35°C. Under this assay condition, 400 units of SU were defined as the amount of enzyme that clot the milk solution in 1 min. Skim milk (10% w/v, Oxoid) containing 10 mM $CaCl_2$ was used as substrate, pre-incubated at 35°C for 5 min. The enzyme extract (0.5 ml) was added to 5 ml of substrate solution, mixed well and incubated at 35°C. The time T (s) referred to as the time period starting from the addition of the enzyme to extract the first appearance of the clots of the milk solution was recorded. MCA was calculated using the following formula:

$$SU = 2400 / T \times S / E$$

Where, T = clotting time (s), S = substrate solution (ml) and E = enzyme solution (ml).

Determination of PA

The method described by Sato et al. (2004) was used to determine the PA of the supernatant. Hammersten casein (1%) was dissolved in 0.1 M Tris - HCL buffer pH 7.5 as substrate and 5 ml of the substrate solution was incubated with 1 ml of crude enzyme solution at 45°C for 30 min; then the enzyme reaction was stopped with 5 ml of trichloroacetic acid mixture (0.11 M CCl_3COOH, 0.22 M CH_3COONa and 0.33 M CH3COOH). After incubation for 30 min, the mixture was filtered using Whatman filter paper No.1, and 2 ml of the filtrate was added to 5 ml of 0.55 M Na_2CO_3 and 1 ml of Folin's reagent (diluted 1:3). The reaction mixture was held at 30°C for 30 min and the optical density at 660 nm was measured using UV-Visible Spectrophotometer (4001/4, Thermo Spectronic, USA). One unit of PA is defined as the amount of enzyme which released 1 µg of amino acid expressed as the tyrosine concentration per min under the above condition.

Protein content

Protein concentration was determined according to the Bradford method using Coomassie reagent (Bio-Rad Laboratories, Hercules, CA, USA) (Bradford, 1976). Bovine serum albumin (Sigma, Germany) was used as standard. The absorbance was measured in the wavelength at 595 nm in a Nano photometer (P- class IMPLEN GmbH - Germany).

Partial purification of MCE by ammonium sulphate precipitate

Solid ammonium sulphate (20 to 80% saturation) was added to a chilled crude enzyme preparation to precipitate the enzyme and carried out at 4°C. The precipitate obtained was collected by centri-

fugation (8000 × *g* at 4°C for 15 min), and dissolved in a minimum quantity of Tris -HCL buffer pH 8.5. This partially purified enzyme was dialyzed using dialysis tubing with molecular cut off 12 000 to 14 000 kDa. MCA and protein content were determined for each fraction.

Gel filtration using Sephadex G-50 fine

Fractions of dialyzed ammonium sulfate with high MCA was lyophilization and then applied to Sephadex G-50 fine column (1.5 × 55 cm) previously equilibrated with 0.2 M phosphate buffer pH 7.0. The enzyme was eluted with the same buffer at a flow rate of 0.6 ml / min. MCA and protein content were determined in each fraction. The fractions that showed MCA were collected and concentrated by lyophilization.

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

The molecular weight of MCE was determined by SDS-PAGE as described by Laemmli (1970) using 4% (w/v) stacking gel and 12.5% (w/v) separating gel. Commassie brilliant blue staining was used to visualize protein bands on the gel. The molecular mass of the enzyme was estimated using a molecular mass markers kit (BioRad).

Influence of nitrogen source on production MCE

The effect of nitrogen source concentration on MCA was determined according to Dutt et al. (2008). The enzyme production media, and incubation conditions were similar to that described above except that different nitrogen sources were used, namely, tryptone soya, casein, tryptophan and trypticase peptone at varying concentrations (0.5, 1, 1.5, 2 and 2.5% w/v). MCA and PA were determined.

Effect of $CaCl_2$ concentration on MCA

Milk substrates supplemented with different concentrations of calcium were prepared using a calcium chloride solution (0.005, 0.01, 0.015, 0.02, 0.025, 0.03 and 0.035 M).

Effects of pH and temperature on MCA and PA

The effect of optimum pH on MCA was determined at 50°C, at different pH values using the following buffers: 0.2 M acetate buffer (pH 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 particular buffer and adjusted to the set pH with 0.1 N NaOH or 0.1 M HCl with fast stirring. For each pH, a control was carried out without the enzyme. The MCA was determined as described above. The PA was determined at 30°C at pH values ranging from pH 5.5 to 8. Using the following buffer solution. 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.

Action of MCE on various milk and casein fractions

The action of the enzyme on casein fractions was evaluated using variable concentrations (0.2 to 2 mg/mL^{-1}) 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.

Data analyses

The analysis of variance was performed to test the effect of nitrogen source, and their interactions on MCA, PA, pH and temperature using the statistical package SAS (SAS Institute Inc, 2010) at the 5% significance level.

RESULTS

Effect of nitrogen source on production of MCE by *P. acidilactici* SH

It was observed that different nitrogen sources (casein, tryptone soya, tryptophan and trypticase peptone) at 0.5 to 2.5% showed significant contribution ($P< 0.001$) on MCE production (Figure 1). Maximum MCA (75 U\ml) was obtained with casein at 0.5% (w/v) compared to other sources evaluated, indicating that casein was the best nitrogen source. Additionally, highest MCA/PA ratio (37.5) was shown by casein compared to tryptone soya, tryptophan and trypticase peptone (Figure 2), while tryptophan showed the lowest value. Further analysis was carried using 5% casein for MCA production.

Purification of the enzyme

Ammonium sulphate precipitation (60%) of crude enzyme extract followed by gel filtration resulted in 727 and 632 SU/ ml, respectively (Table 1). The partial purified enzyme showed a single band using 12.5% SDS-PAGE gel, with a molecular weight of approximately 29 kDa (Figures 3 and 4).

Effect of $CaCl_2$ on MCA and PA

Calcium had a positive effect on the MCA as shown in (Figure 5). Maximum MCA of 77 SU/ml was achieved at 0.02M $CaCl_2$ concentration, but decreased to 19 SU\ml at 0.035M $CaCl_2$.

Effect of temperature and pH on MCA, PA and MCA/PA

The MCA and MCA/PA were assayed at different temperatures ranging from 25 to 60°C at constant pH of

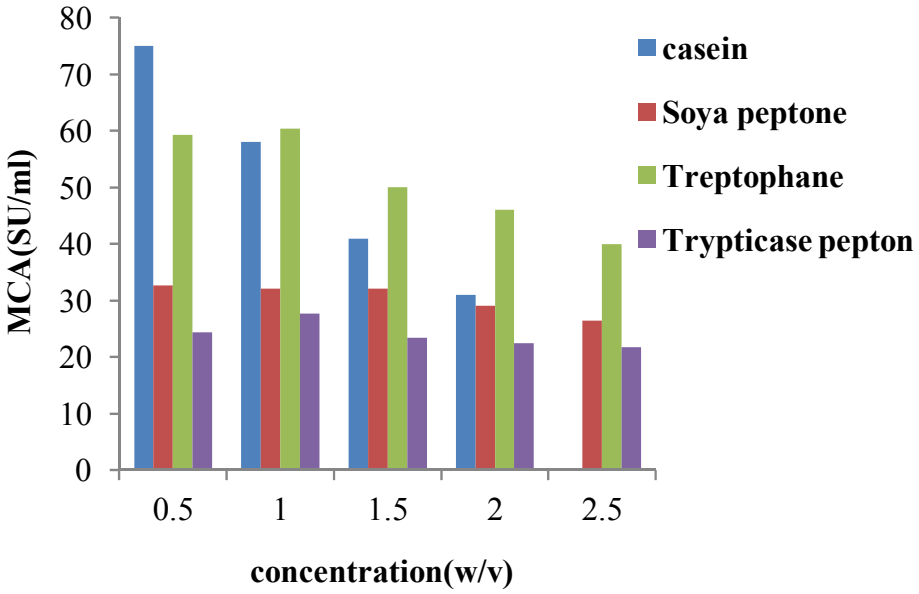

Figure 1. Effect of organic nitrogen source on MCA at 50°C.

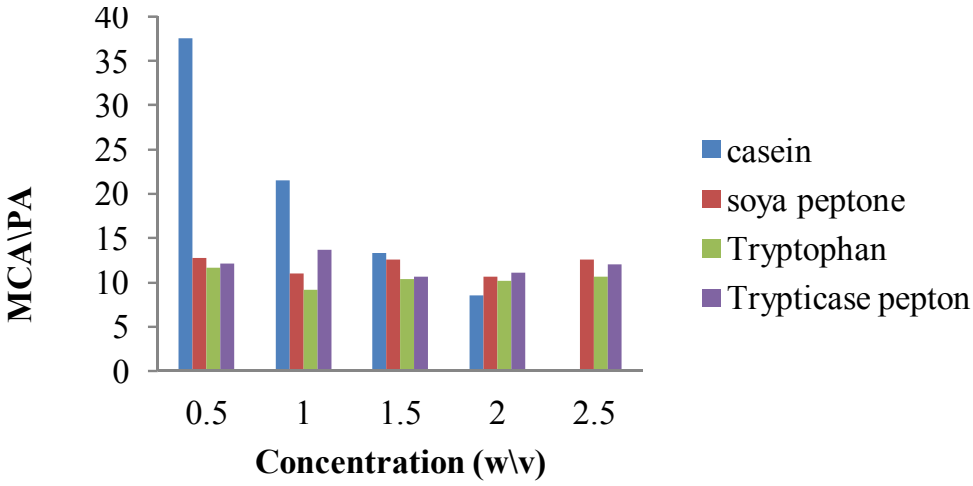

Figure 2. Effect of organic nitrogen source on MCA/ PA at 50°C.

Table 1. Purification steps of MCE produced by *Pedioccous acidilactici.*

Purification steps	MCA(SU\ml)	Protein content(mg\ml)	Specific activity(u\mg)	Fold	Yield %
CE	750	6.5	115.4	1.00	100
60% Ammonium sulphate	727	4.7	145.6	1.40	97
Sephadex G-50 fine	632	2.5	258.8	2.3	84

6.0. The MCA for the enzyme produced by *P. acidilactici* SH was active between 30 to 55°C. The optimal temperature for MCA and MCA/PA was 50°C with values 75 and 37.5 SU/ml, respectively (Figure 6). The MCA and MCA/PA decreased to 46 and 15 SU/ml, respectively, after incubation at 55°C and deactivated completely after 60°C during the 15 min reaction time.

Changing the pH from 6.0 to 8.0 MCA and MCA/PA

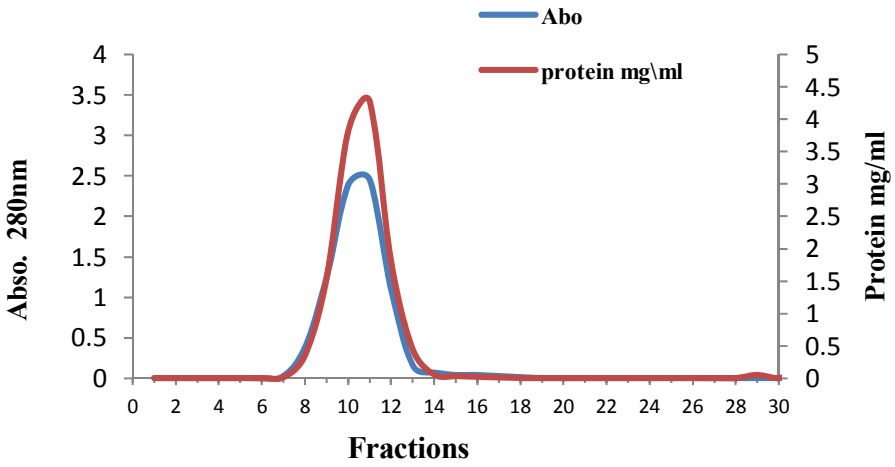

Figure 3. Gel filtration by using Sephadex G-50 fine.

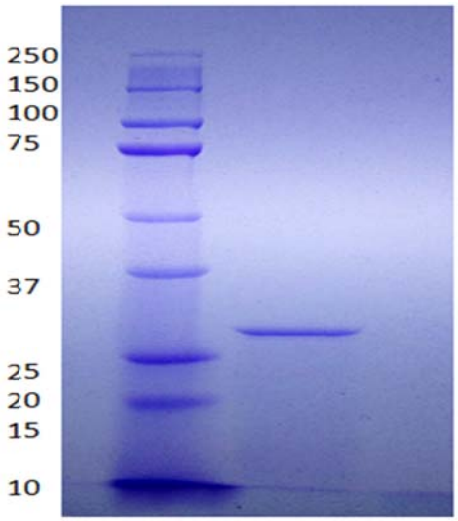

Figure 4. SDS 12.5%PAGE**of** the purified enzyme from SH strain.

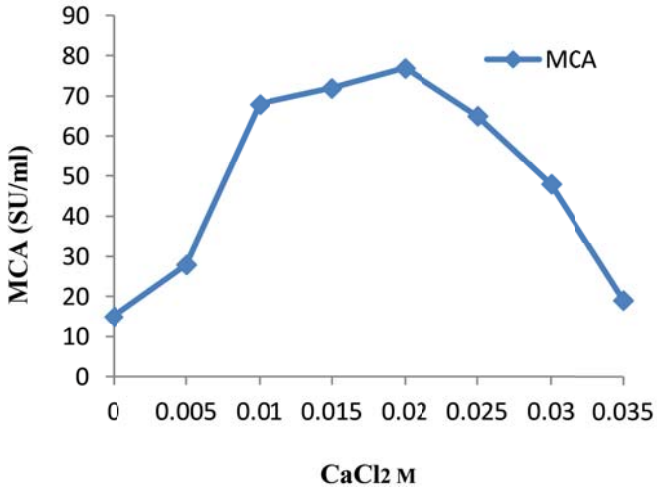

Figure 5. Effect of $CaCl_2$ on MCA.

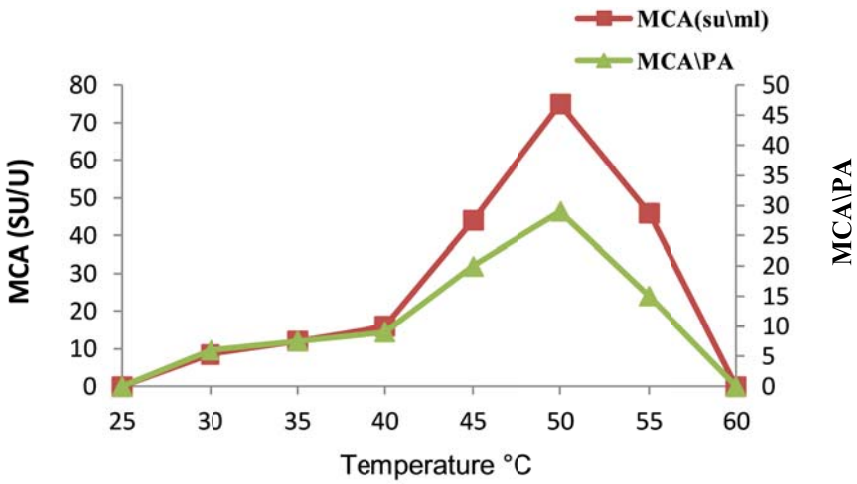

Figure 6. Effect of temperature on MCA by SH enzyme.

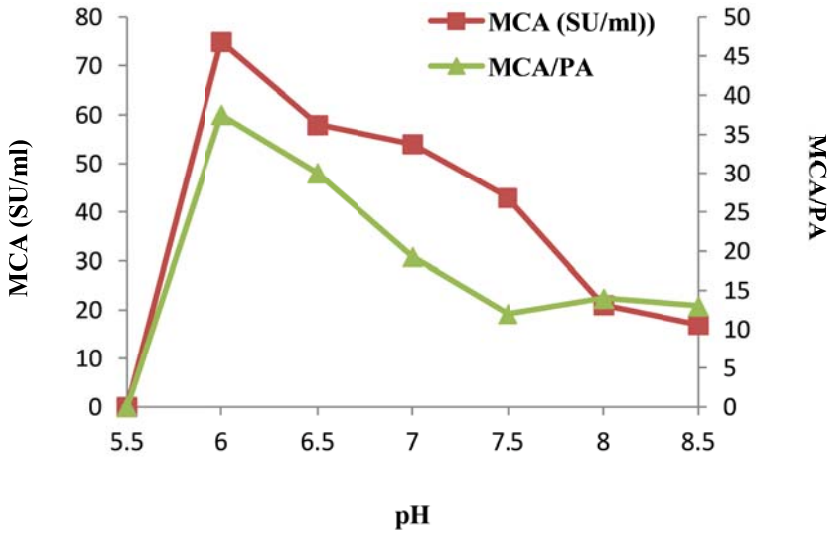

Figure 7. Effect of pH on MCA by SH enzyme at 50ºC

was significantly affected (P<0.001) when the enzyme activity was evaluated at 50°C (Figure 7). Results show that at pH 6.0 and 50°C, MCA was maximum (75 SU/ml) and the MCA / PA was highest (37.5). Increasing pH 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 were 30, 19, 12, 14 and 13, respectively.

The PA was assayed at different temperatures ranging from 25 to 60°C at constant pH of 6.0 for 30 min (Figure 8). PA increased with temperature within the range of 30 to 55°C with maximum PA and MCA/PA 3.6 and 15 U/ml, respectively. The PA value increased gradually with the highest value of pH 7.5 (3.6 U/ml) and MCA/PA (12) after that PA decreased to 1.3 U/ml at pH 8.5, when incubated at 50°C (Figure 9). These results clearly indicate that the enzyme is a neutral enzyme.

Action of the purified enzyme on casein fractions

It was observed that alpha and beta casein was not hydrolyzed by the PA of enzyme as indicated by urea SDS-PAGE (Figure 10). However, κ-casein was hydrolysed by enzymes as indicated by loss of band within 10 min. A new band with approximate MW between 10,000 to 11,000 kDa was detected.

DISCUSSION

The MCAs of microorganism have different characteristics and values. Fungi especially *Mucor, Rhizopus, Endothia, Rhizomucor,* and *Aspergillus* have been used for production of MCE. Some *Bacillus* sp.,

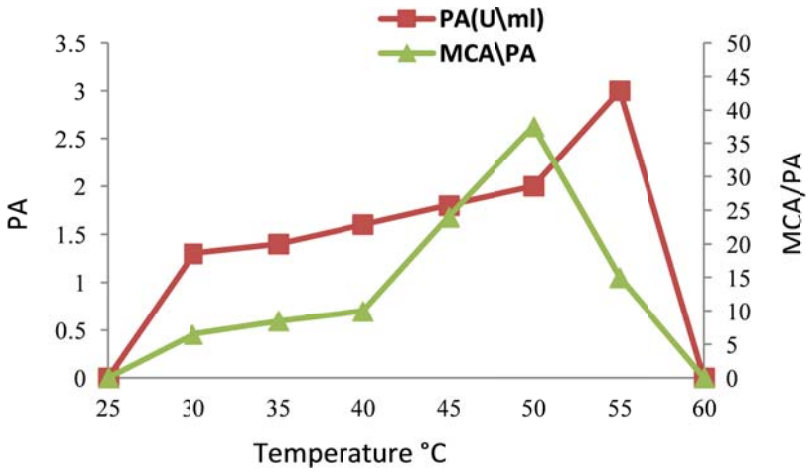

Figure 8. Effect of temperature on PA by SH enzyme.

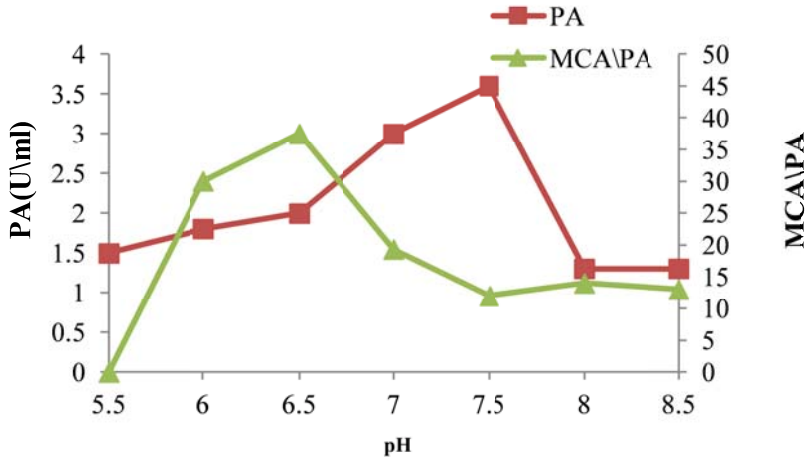

Figure 9. Effect of pH on PA by purified enzyme.

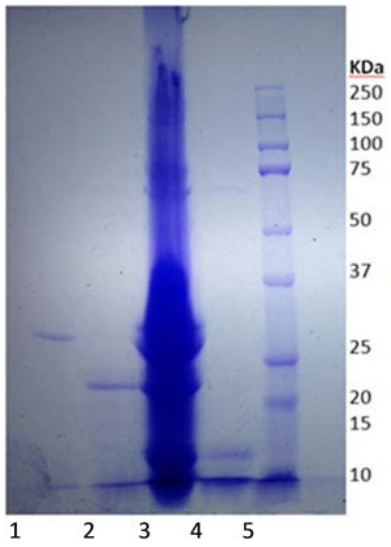

Figure 10. Action of the enzyme on casein fractions, SDS urea page 10% gel α, β and ķ- casein hydrolysis by the enzyme from *pediococcus acidilactici* SH. Lane 1 β -casein; lane 2 α- casein; lane 3 whole casein; lane 4 k-casein; lane 5; Molecular weight protein markers.

notably, *B. subtilis, B. cereus, B. licheniformis,* (Srinivasan and Dhar, 1990; D'Souza and Pereira 1982) and others like *Myxococcus xanthus* strain 422 (Poza et al., 2003) and *Nocardiopsis* (Cavalcanti et al., 2005) were reported to produce an enzyme with MCA. LABs have also been reported as MCE producers such as *L. helveticus* (Hebert et al., 2001). Four lactic acid bacteria from 157 isolates isolated from various fermented foods such as *miso*, soy sauce, and yogurt produces MCE (Sato et al. (2004).

In the microbial fermentation process, optimization of culture media is important to increase the amount of MCE. Nitrogen sources influenced the MCA and MCA/PA of MCE of microorganisms. In this study the LAB identified as *P. acidilactici* SH isolated from "Belacan" showed highest MCA (75 SU/ml) using 0.5% casein and decrease at higher casein concentrations (1-2.5% w/v). This value was higher than the MCA obtained from *E. faecalis* TUA2495Lwas (21 SU/ml) when casein was used as a nitrogen source in production media (Sato et al., 2004). However, Dutt et al. (2008) reported that maximum MCA (120 SU/ml) from *B. subitls* when 1.5% casein was used as nitrogen source and decreased MCA to (81 U/ml) when increase the concentration of casein to 2%.

In addition casein played an important role in microbial rennet production under both solid state fermentation and submerged fermentation conditions in the case of *R. miehei* (Silveira et al., 2005). Maximum MCA (130 SU/ml) was obtained from *Mucor mucedo* DSM 809 that was grown in enzyme production media, containing 0.5% (w/v) casein (Yegin et al., 2010). In contrast *R. nainitalensis* showed maximum MCA obtained when 1.5% (w/v) casein was used as a nitrogen source in the production media. Khademi et al. (2013) and De-Lima et al. (2008) reported that for rennet obtained from *M miehei* NRRL 3420, the highest MCA was obtained when 0.4% casein was used and decreased MCA when casein concentration was increased to 0.8% in enzyme production media. The MCA and PA of *M miehei* NRRL 3420 can be affected by nitrogen sources (Moon and Parulekar, 1991; Chu et al., 1992; Sato et al., 2004; Patel et al., 2005). The ratio of MCA/PA can be used as an index to substantiate the sufficiency of MCE to be used as calf rennet substitute (Merheb et al., 2010).

The purification of the enzymes by ammonium sulphate remains a common use, for the concentration of the enzymes from microbes (Lee et al., 2002; Cheng et al., 2010) because of its high solubility, cheaper cost and stabilizing effect on most enzymes as well as can be used in acidic and neutral pH solutions (Rifaat el at., 2005). The crude MCE from *P. acidilactici* SH was purified by ammonium sulphate (60% saturation) and gel filtration by using Sephadex G-50 fine; the purified MCE showed a single band of molecular weight 29 kDa by SDS-PAGE 12.5% wit maximum specific activity (258.8 U/mg) (Table 1). Wu et al. (2013) observed two major bands between 25 and 30 kDa present in MCE from *B. natto*, and 30 KDa from *Brassica napus* Seeds (El-Sayed et al., 2013); but slightly different from the band produced by *E. faecalis* TUA2495L with 34 KDa, as reported by Sato et al. (2004) and *L. casei*- D1-1 with 35 KDa as reported by Xing et al. (2012).

Generally, milk clotting as results of enzyme activity could not be achieved below pH 5.5 because the protein in skim milk coagulated at low pH values even in the absence of the enzyme. In this study, the purified enzyme from *P. acidilactici* SH showed the highest activity at pH 6.0 (MCA 75 SU/ml) followed by a decrease in MCA at higher pH values indicating the enzyme is a neutral enzyme and consider suitable for dairy product. The results show that *P. acidilactici* SH is ideal for the production of the MCE as rennet that can be used in some dairy products (gel formation), and a good choice for further studies and industrial exploitations. Quite similar behavior was reported for the crude extract from *E. faecalis* TUA2495L studied by Sato et al. (2004) which exhibited the optimum MCA at pH 5.8 and also that reported by Merheb et al. (2010) who found that pH 5.7 was the optimum MCA of the precipitate extract from *P. citrinum.* PA of *P. acidilactici* SH increase from pH 5.5 until it reached a maximum reaction rate at pH 7.5 then lost activity at pH 8.5. In contrast, proteolytic enzymes from fungi generally exhibit maximum PA 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 was shown to have weak activity in alkaline conditions (He et al., 2011).

The highest MCA (75 SU/ml) was exhibited by *P. acidilactici* SH at 50°C and no activity at 60°C. Similarly, Vishwanatha et al. (2010) reported that MCE from *A. oryzae* MTCC 5341 was active 55°C and no activity was found when the temperature was 62°C. In contrast, Sato et al. (2004) reported that the MCE activity of *E. faecalis* TUA2495L was optimum at 70°C.

Calcium plays an important role in milk coagulation as well as in the gel formation. Increasing the concentration of $CaCl_2$ over 10 mM may have a negative effect on curd formation, due to the additional calcium which will increase the positive charge on the surface of the micelle, causing charge dissonance and produces weaker gel or no gelation at all (Sandra el at., 2012). In the present study MCA increased from 28 to 77 SU/ml when CaCl2 was included at 0.005 to 0.02 M; MCA decreased at higher concentration of CaCl2. Verma et al. (2012) reported that the increasing calcium concentration to 20 mM in goat or cow milk decrease milk clotting time. Vairo-Cavalli et al. (2005) suggested that the increase Ca_2^+ concentration in the substrate increase the ionic force or the saturation of negative residues of the casein micelles. The MCA of the *P. acidilactici* SH enzyme completely hydrolysed k-casein but slightly hydrolyzed α-casein but not β casein as shown by urea-SDSPAGE (Figure 10).

Similar observation was reported by Sato et al. (2004) of which the enzyme produced by *E. feacalis* 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 et al., 2001). However, extracted enzyme from *T. aurantiacus* hydrolysed β-casein even after 60 min of incubation studied (Merheb et al., 2007) not for producing curd but for the production of bioactive peptides. This selective hydrolysis of casein fractions are very important for cheese making to produce high yield curd.

Conclusion

This study demonstrates that the casein as nitrogen sources produce proteolytic enzymes that produced high MCA with a low PA which hydrolyzed k-casein. *P. acidilactici* SH isolated from local fermented foods can be a source of enzyme for cheese production.

Conflict of Interests

The author(s) have not declared any conflict of interest.

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