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PRODUCTION AND PARTIAL CHARACTERIZATION OF A MILK-CLOTTING PROTEINASE PRODUCED BY *BACILLUS SUBTILIS* SMDFS-2B MN715837 IN SUBMERGED CULTURES

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ABSTRACT

This study focused on the production and partial characterization of a milk-clotting protease produced by *Bacillus subtilis* SMDFS 2B in submerged cultures, under partially optimized conditions. The crude enzyme was recovered in the culture supernatant and concentrate was produced after cell removal and subsequent dialysis. Inhibition studies were conducted employing four distinct protease inhibitors: Pepstatin-A, Phenylmethane-sulphonyl-fluoride (PMSF), Ethylenediaminetetraacetic acid (EDTA), and iodoacetamide (IA). The effect of temperature, pH, metal ions and substrate concentration on milk-clotting activity were also evaluated. The thermal stability of the enzyme was determined by incubating the crude enzyme at a temperature value ranging from 35 ^{o}C to 60 ^{o}C. Similarly, pH stability was determined at pH values ranging between 4.5 and 8.0. The highest milk-clotting activity was observed at a temperature of 55 ^{o}C and pH 5.5. The crude enzyme preparation remained stable on incubation at 35 ^{o}C and 40 ^{o}C for 15 min and at pH 5.5. The enzyme also showed the lowest residual milk-clotting activity in the presence of EDTA (7.94%) and Pepstatin-A (26.71%). The addition of Mg^{2+} and Mn^{2+} significantly increased milk-clotting activity. The enzyme also showed an elevation in its apparent milk-clotting activity upon increasing the substrate (skim-milk) concentration. Thus, the milk-clotting protease produced by *B. subtilis* SMDFS 2B by submerged fermentation revealed some interesting milk-clotting characteristics. This may open the way for applications in the food and dairy industries.

Keywords: *Bacillus subtilis*, Characterization, Inhibition, Milk-clotting activity

INTRODUCTION

Calf chymosin (EC3.4.23.4) is widely used as a milk-clotting enzyme (MCE) in cheese production. However, a decrease in the global supply of calf rennet versus the increasing demand of chymosin in the production of cheese necessitates the exploration for potential substitutes **(Hang et al., 2016)**. Milk-clotting enzymes from microbial sources have been discovered and developed to replace calf rennet (Ding et al., 2012).
Several microorganisms produce MCEs, with the MCEs produced by fungi such as *Rhizomucor miehei*, *Rhizomucor pusillus* var. Lindt, *Aspergillus oryzae* and *Endothia parasitica* widely used in cheese production **(Wu et al., 2013).** Currently, researches on the production of milk-clotting enzyme from bacteria are less as compared to those from fungi **(Ding et al., 2012).** However, there exist some wild-type bacteria that produced milk-clotting enzymes with substantial milk-clotting activity (MCA) under submerged fermentation conditions **(Dutt et al., 2008).**
Bacteria of genus *Bacillus* are known for the production of large quantities of extracellular proteases and other types of enzymes for industrial use (Ageitos et al., 2007). In particular, the well-researched *Bacillus subtilis* is known to secrete several enzymes of industrial importance (Kumari et al., 2016). The present study describes the production and partial characterization of a milk-clotting proteinase from *Bacillus subtilis* SMDFS-2B that was isolated by our laboratory.

MATERIALS AND METHODS

Strain and Cultivation Conditions

The bacterium used in the present study was isolated from soil collected from Samara Ethiopia (11°48'09.3"N 40°59'26.8"E) and identified as *Bacillus subtilis* SMDFS-2B using 16S rRNA methodology **(Guleria *et al*., 2016).** The culture medium as employed by us contained: galactose (20 g/L), wheat bran (30 g/L), sodium chloride (5 g/L), magnesium sulfate ($MgSO_4 \cdot 7H_2O$, 5 g/L), potassium phosphate (KH_2PO_4, 2 g/L) and calcium carbonate (3 g/L). Final pH was adjusted to 5.5. The culture medium was sterilized by autoclaving at 121 ^{0}C for 20 min. Subsequently, the media was inoculated with 4% inoculum suspension ($2.5*10^8$ cells per mL) and incubated for 72 h using an orbital shaker (150 rpm) at 35 ^{0}C **(Ding et al., 2012).**

Crude enzyme preparation (Dialysis)

Samples were collected after 72 h under aseptic conditions and centrifuged at 10,000 rpm (MaxQ 2000 Open-Air Platform Shaker, Thermo Fisher Scientific, USA) for 10 min. The supernatant was collected by decantation and tested for milk-clotting activity **(Ding et al., 2012).** The clarified culture supernatant was subjected to dialysis using 8-10 cm of dialysis membrane (10 kDA cut-off) immersed in 100 ml of 2% sodium carbonate solution and kept in the refrigerator at 4 ^{0}C. The liquid crude enzyme extract was poured in to a membrane tied with rubber tightly on one side and then the other side of the membrane was tied again with rubber. A powder of carboxymethyl cellulose salt was added to the membrane and the membrane was spread over a flat plate for overnight. Finally the residual protein in the membrane was collected from membrane by dipping with sodium phosphate buffer (20 mM, pH=6.0) **(Ramachandran and Arutselvi, 2013).**

Protein determination

Total protein was determined using Bicinchonic acid (BCA) method **(Walker, 2002)** using bovine serum albumin (BSA) (Thermo Scientific^{TM}) as a standard.

Milk-clotting Assay

Milk-clotting activity was performed according to **Arima et al., (1970).** Briefly, 0.1 mL of the crude enzyme was added to 1.0 mL of reconstituted skim-milk (Nestle^{TM}) in 5.0 mL test tubes that were pre-incubated at 35 ^{0}C for 10 min. The enzyme substrate consisted of 10 g dry skim-milk/100 mL in 0.01 M $CaCl_2$ (AppliChemTM). The appearance of the first clotting flakes was visually evaluated and the activity quantified in terms of Soxhlet Units (SU). The endpoint was recorded when discrete particles were discernible. The clotting time T (s), the period from the addition of crude enzyme to the appearance of the first clots was employed to calculate the clotting activity using the following formula:

$$SU = (2400 * 1 * D)/(T * 0.1)$$

Where T = clotting time (s) and D= dilution of crude enzyme preparation
One SU is expressed as the quantity of enzyme required to clot 1.0 mL of a solution comprising 0.1 g skim-milk powder and 0.01M calcium chlorides at 35 ^{0}C within 40 min.

Proteolytic activity Assay

The proteolytic activity was assayed according to **Arima et al., (1970).** Thus, 0.5 mL of the enzyme extract was added to 2.5 mL of 1% (w/v) soluble casein in 20 mM potassium phosphate buffer at pH 6.5, and the mixture was incubated in a water bath at 35 ^{0}C for 10 min. After having added 2.5 mL of 0.44 M trichloroacetic acid to terminate the reaction, the mixture was filtered through Whatman No.1 filter paper. The filtrate was then mixed with 1.0 mL volume of three times diluted 2N Folin-Phenol reagent and 2.5 mL of 0.55 M sodium carbonate solution. The mixture was incubated at 35 ^{0}C for 20 min and optical density (OD) was measured with a spectrophotometer (UV-Vis, Liantrinsat, Model-CF728YW-UK) at 660 nm. One unit (1 U) of enzyme activity was

defined as the amount of enzyme that liberated 1µg of tyrosine per mL in one minute.

$$\text{PA (U/mL)} = \frac{\mu\text{Tyr} * V_t}{V_s * T * V_a}$$

Whereas PA: Protease activity, µTry: µg of tyrosine equivalent released, Vt: Total volume of assay in mL (5 mL of substrate plus 1 ml of Enzyme plus 5 ml of TCA), Vs: Sample volume (ie. The volume of protease used for assay in mL), T: reaction time (i.e Time of incubation in minutes, 10 min), Va: Volume of assayed (i.e the final volume of the product used in calorimetric determination)

Inhibition Studies

The effects of known protease inhibitors on the milk-clotting activity of the dialyzed enzyme were examined using cysteine protease inhibitor, iodoacetamide (1mM and 10mM); aspartic protease inhibitor, pepstatin A (0.02mM, 0.04mM, 0.6mM, 0.08mM and 0.1mM); metalloprotease inhibitor, EDTA (5mM, 10mM); and serine protease inhibitor, phenylmethane sulphonyl fluoride (PMSF) (1mM, 10mM). After the addition of inhibitors into the enzyme samples, the mixtures were incubated at 35 ^{0}C for 30 min., the residual milk-clotting activity was then assayed. Residual activity was defined as the percentage of the activity determined in the absence of inhibitors **(Yegin et al., 2012).**

Determination of optimum temperature and thermal stability

For the determination of the optimum temperature of the dialyzed enzyme, the milk-clotting activity of the reaction mixture (10 % skim milk (w/v) in 0.01 M $CaCl_2$) was assayed at different temperature (25, 30, 35, 40, 45, 50, 55, 60, 65 and 70 ^{0}C). Likewise, the thermal stability was determined by incubating the crude enzyme at a temperature in the range of (35, 40, 45, 50, 55 and 60 ^{0}C) for 15 and 30 min. After incubation, the samples were cooled in an ice bath prior to assay for determination of residual milk clotting activity **(Yegin et al., 2012).**

Determination of optimum pH and pH stability

The substrate (skim milk) containing 0.01M $CaCl_2$ was prepared at pH (4.5, 5.0, 5.5, 6.0) using 20 mM citrate-buffer, while pH (6.5, 7.0, 7.5 and 8.0) solutions were prepared using 10 mM potassium phosphate buffer. Then the optimum pH of the dialyzed enzyme was determined by carrying out the milk-clotting assay according to section 2.4. The pH stability was determined by incubating the dialyzed enzyme sample in 20 mM citrate buffer having pH (4.5, 5.0, 5.5, and 6.0) and in 10 mM potassium phosphate buffer (6.5, 7.0, 7.5 and 8.0) at 25^0 C for 30 minutes. After incubation, the samples were examined for relative milk-clotting activity **(Ageitos et al., 2007; Yegin; et al., 2012)**

Effect of substrate concentration on the milk-clotting activity

To determine the effect of substrate concentration on the milk-clotting activity of the dialyzed enzyme, the skim-milk solution with different concentration (25 g/L, 50 g/L, 100g/L, 150 g/L and 200 g/L) were prepared and the standard milk-clotting activity was determined as previously described in section 2.4. The highest activity obtained was taken to be 100% **(El-Tanboly et al., 2013).**

The impact of additives on the milk-clotting activity

The effect of various metal ions (Na^+, K^+, $Ca^{2+,}$ Mg^{2+} Mn^{2+}, Fe^{2+}, Zn^{2+}, Ni^{2+}, $Cu^{2+,}$ and Co^{2+}) on the milk-clotting activity was studied. First the metal salts in the forms of chloride and sulfate were added in the skim-milk substrate at a final concentration of 10 mM and then the milk-clotting assay was conducted after 30 minutes (Table 1). For comparison, the control (without the addition of any metal ion) was taken or set as 100% and the effect of various metal ions was expressed as or relative activity (Kumari et al., 2016).

Table 1 Metals ions used for milk-clotting assay

S.No	Type of Metal Ions	Concentrations
1.	Control (without adding any metal ion)	-
2.	NaCl	10 mM
3.	KCl	10 mM
4.	$CaCl_2$	10 mM
5.	$MgCl_2$	10 mM
6.	$FeCl_2$	10 mM
7.	$MnCl_2$	10 mM
8.	$NiCl_2$	10 mM
9.	$MgSO_4.7H_2O$	10 mM
10.	$MnSO_4.7H_2O$	10 mM
11.	$ZnSO_4.7H_2O$	10 mM
12.	$CuSO_4.7H_2O$	10 mM
13.	$FeSO_4.7H_2O$	10 mM
14.	$CoSO_4$	10 mM

Effect of $MnSO_4$ and $CaCl_2$ concentration on the milk-clotting activity

To study the effect of $MnSO_4$ and $CaCl_2$ on the clotting efficiency of the dialyzed enzyme, various concentrations of $MnSO_4$ and $CaCl_2$ (0.00 M, 0.005 M, 0.01M, 0.05M, 0.1M, and 0.2M) were incorporated in the reaction mixture (Table 2). Time taken for the appearance of the first clot was recorded and compared with the control sample **(El-Tanboly *et al*., 2013).**

Table 2 Concentration of $MnSO_4$ and $CaCl_2$ used for milk-clotting assay

S.No	Conc. Of $MnSO_4$ in mM	Conc. of $CaCl_2$ in mM
1	0.00	0.00
1.	5.00	5.00
2.	10.00	10.00
3.	50.00	50.00
4.	100.00	100.00
5.	200.00	200.00

Data analysis

Data analyses were performed using SAS software version 9 (Inc. Cary NC USA). The experiments were carried out in triplicate. Analysis of variance (ANOVA) and means comparisons were done by Duncan's multiple range tests.

RESULTS

Crude enzyme production and partial purification

Bacillus subtilis SMDFS 2B was cultivated in shaking flasks under submerged fermentation conditions utilizing a simple media containing wheat bran, under partially optimized conditions (Table 3). The maximum milk-clotting activity (312 U/mL) was recorded in the culture supernatant after 72 h of fermentation. The culture supernatant was dialyzed and concentrated to yield a crude enzyme preparation. The milk-clotting activity was increased 2.0-fold after dialysis (Table 4).

Table 3 Production of crude enzyme from *B. subtilis* SMDFS2B under SmF

Parameters	Measurement Unit
Initial media pH	5.5
Inoculum size	4% of ($2.5*10^8$ Cells/mL)
Fermentation time	72 h
rpm	150
MCA	311.74±4.05 U/mL
PA	231.04±5.18 U/mL

rpm: revolution per minute, MCA: Milk-clotting activity (U/mL), PA: protease activity (U/mL)

Table 4 MCA, specific activity and concentration fold of dialyzed protease from *B.subtilis* SMDFS 2B

Sample	MCA (U/mL) Mean ±SD	Protein conc. (mg/mL)	Specific activity (U/mg)	Purification Fold
Crude enzyme	311.74±4.05[b]	17.67	17.64	1
Dialyzed enzyme	406.90±6.90[a]	11.13	36.56	2.07

MCA: Milk-clotting activity (U/mL), specific activity: MCA (U/mL)/Total protein (mg/mL), SD: standard deviation, Mean: is average of three measurements, Different letters (a, b,) designate significantly different means as determined by Duncan multiple mean comparison test (P<0.05).

Inhibition study

The type of milk-clotting proteinase produced by *B. subtilis* SMDFS 2B was determined by conducting inhibition experiments using four protease inhibitors. Incubation of the enzyme with individual protease inhibitors showed a significant reduction in milk-clotting activity. Residual milk-clotting activity values of 27%, 43%, 7.9% and 37% were found when using Pepstatin A (0.1 mM), Iodoacetamide (10 mM), EDTA (10 mM) and PMSF (10 mM), respectively (Table 5).

Table 5 Inhibition study of the dialyzed enzyme from *B.subtilis* SMDFS 2B

Inhibitors	Conc. in mM	MCA (U/ml) mean±SD	Residual MCA (%)
Pepstatin A	0.02 mM	279.11±3.25[b]	68.59
	0.04 mM	193.30±10.09[d]	47.51
	0.06 mM	160.58±2.69[fg]	39.46
	0.08 mM	147.13±10.77[g]	36.16
	0.1 mM	108.68±2.95[h]	26.71
Iodoacetamide	1 mM	214.30±1.91[c]	52.67
	10 mM	175.19±1.28[e]	43.06
EDTA	5 mM	177.12±0.65[e]	43.53
	10 mM	32.32±0.02[i]	7.94
PMSF	1 mM	161.54±8.67[f]	39.70
	10 mM	150.47±0.47[fg]	36.98
Control (dialyzed Enzyme)	-	406.90±6.90[a]	100.00

ND: Not determined, MCA: Milk-clotting activity (U/mL), Pep A: Pepstatin A, I.A: Iodoacetamide, EDTA: ethylenediaminetetraacetic acid, PMSF: phenyl-methane sulphonyl fluoride, SD: standard deviation, Mean: is average of three measurements, different letters (a, b, c, d, e, f, g) designate significantly different means as determined by Duncan multiple mean comparison test (P<0.05).

Effects of temperature on enzyme activity and stability

In determination of the optimum temperature for the dialyzed enzyme, the milk-clotting activity was assayed at the temperature range from 25 ^{o}C to 70 ^{o}C using skim-milk at pH 6.2. The highest milk-clotting activity (2833 U/mL) for the dialyzed milk-clotting protease from *B. subtilis* SMDFS-2B was obtained at 55 ^{o}C. Thereafter, the milk-clotting activity of the enzyme drastically decreased and insignificant activity was observed at 70 ^{o}C (Fig 1). Thermal inactivation experiments were conducted at a temperature ranges from 35 ^{o}C to 60 ^{o}C using skim-milk at pH 6.2. The dialyzed enzyme from *B. subtilis* SMDFS-2B showed stability on incubation at 35 ^{o}C and 40 ^{o}C for 15 min by exhibiting a residual activity of 81% and 74 %, respectively. However; the residual activity of the enzyme preparation slightly decreased to 55% after incubating at 60 ^{o}C for 15 min. Further increase in incubation time to 30 min drastically reduced the milk-clotting ability of the enzyme (Fig. 2).

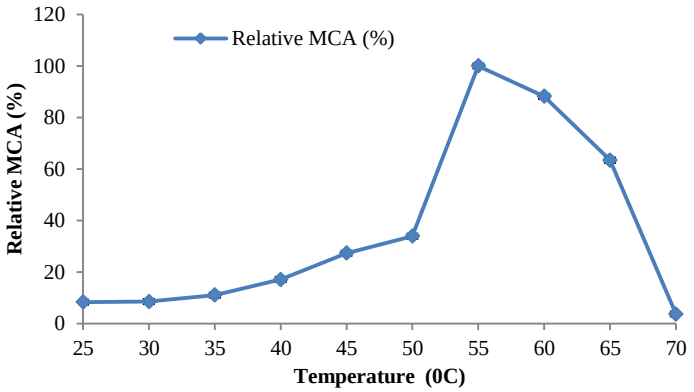

Figure 1 The effect of temperature on the activity of milk-clotting protease produced by B. subtilis SMDFS 2

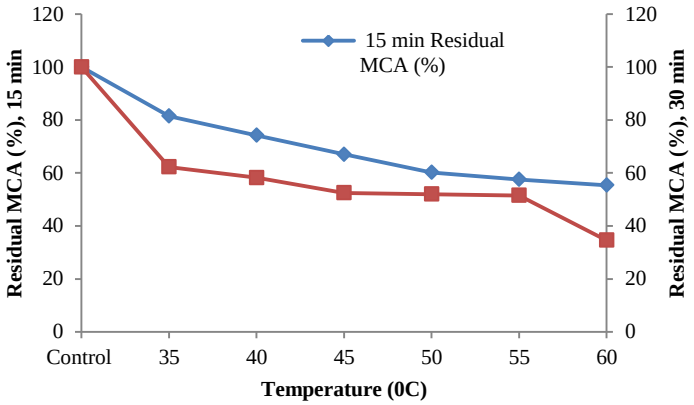

Figure 2 The effect of temperature on the stability of milk-clotting protease produced by B. subtilis SMDFS2B

Effect of pH on enzyme activity and stability

The effect of pH on the milk-clotting activity of the dialyzed enzyme was studied at pH ranges from 4.0 to 8.0 at 35 ^{o}C. The enzyme exhibited the maximal rate of reaction at a pH range (4.0-6.0) as shown in Fig. 3. However, the highest milk-clotting activity (375 ± 6 U/mL) was obtained at pH 5.5. The stability of the dialyzed enzyme at different pH values is shown in Fig. 3. The enzyme showed maximum stability at pH 5.5. As the pH becomes extreme to either side, the activity of the enzyme decreased significantly.

Effect of skim-milk concentration on enzyme activity

The effect of skim-milk concentration (25 g/L – 200 g/L) on the milk-clotting activity of the dialyzed enzyme is shown in Fig. 4. The enzyme showed an elevation in its milk-clotting activity with increasing skim-milk concentration (Fig. 4). The MCA reached a maximum when the skim-milk concentration was increased to 200 g/L.

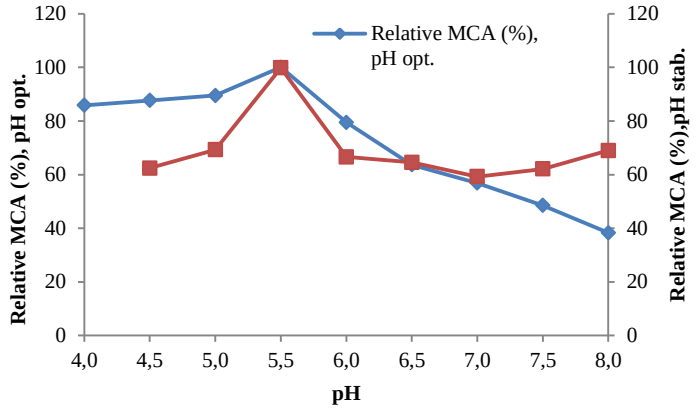

Figure 3 The effect of pH on the activity and stability of milk-clotting protease produced by B. subtilis SMDFS 2B

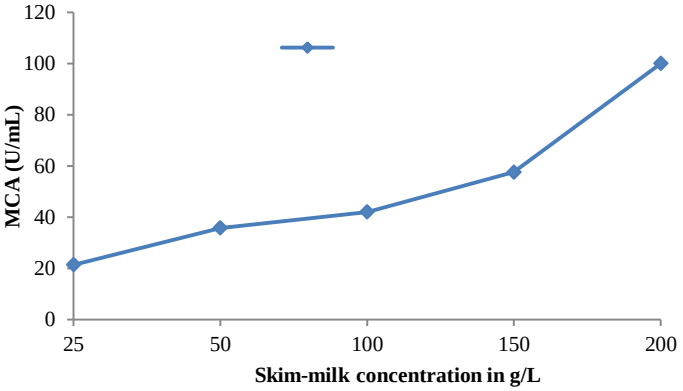

Figure 4 The effect of skim-milk concentration on the activity of milk-clotting protease from B.subtilis SMDFS 2B

Effect of metal ions on enzyme activity

The effect of various monovalent and divalent ions on the milk-clotting activity of the dialyzed enzyme from *B. subtilis* SMDFS-2B was studied at 10 mM concentration (Fig. 5). Na^{+}, K^{+}, and Zn^{2+} had a slight stimulatory effect on milk-clotting activity, whereas $Cu^{2+,}$ Co^{2+}, and Ni^{2+} significantly inhibited the milk-clotting activity. In contrast, Mg^{2+} and Mn^{2+} had a significant stimulatory effect on the milk-clotting activity of the crude enzyme from *B. subtilis* SMDFS-2B.

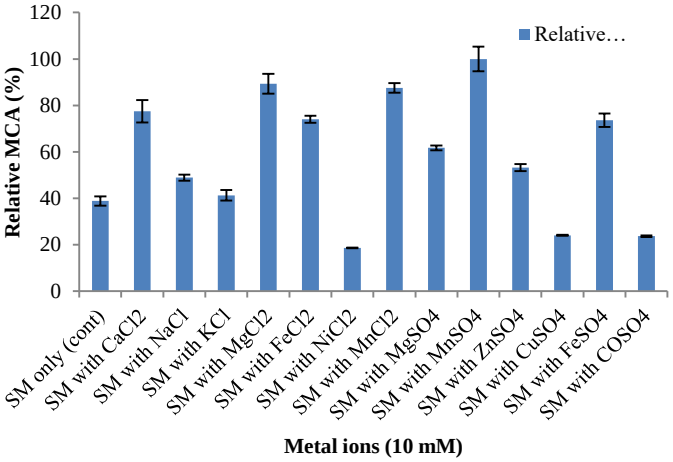

Figure 5 The effect of metal-ions on activity of milk-clotting protease from B.subtilis SMDFS 2B

Effect of $CaCl_2$ and $MnSO_4$ concentration on enzyme activity

The effect of $CaCl_2$ and $MnSO_4$ concentration on the milk-clotting activity of the dialyzed enzyme from *B. subtilis* SMDFS-2B was studied in the range from 5 mM to 200 mM. The highest milk-clotting activity was obtained at 10 mM and 50 mM concentration of $MnSO_4$ and $CaCl_2$, respectively (Fig. 6). The clotting time increased with increasing concentration of Ca^{2+} and reached the maximum at 50mM and thereafter declined severely. The milk clotting activity of the crude enzyme also increased with increasing concentration of Mn^{2+} and reached highest at 10 mM and reduced sharply afterward.

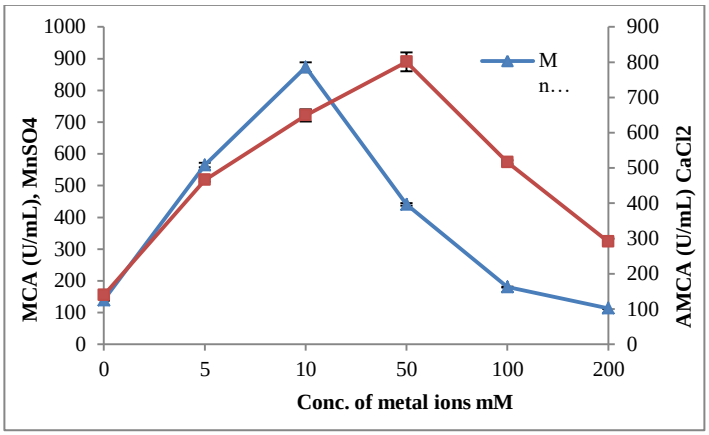

Figure 6 The effect of $MnSO_4$ and $CaCl_2$ concentration on the activity of milk-clotting protease produced by B.subtilis SMDFS 2B

DISCUSSION

The milk-clotting activity of the crude enzyme preparation (312 ± 4 U/mL) obtained from *B. subtilis* SMDFS-2B in the present study was higher when compared to the activity recorded for crude milk-clotting protease from *Thermomucor indicae-seudaticae* N31 (160 ±12 U/mL) **(Merheb-Dini *et al.*, 2010)** and from *Bacillus amyloliquefaciens* SP1(130 U/mL) **(Guleria et al., 2016).** However, a higher activity (1190 U/mL) was obtained from an indigenous *Bacillus subtilis* strain after the production media was optimized using response surface methodology (RSM) **(Dutt *et al.*, 2009).** The variation recorded in milk-clotting activity could be due to the differences in microbial strain and/or media used for the enzyme production.

Dialyzing the crude enzyme resulted in an increase in the milk-clotting activity of the preparation by 2-fold. This may be due to the removal of low-molecular-weight interfering or inhibitory compounds. Similarly, partial purification of a milk-clotting protease from *M. pusillus* **(Nouani *et al.*, 2011)**, a serine alkaline protease from *B. mojavensis* **(Beg and Gupta, 2003)** and mixed proteases from *B. amyloliquefaciens* **(Cho *et al.*, 2002)** using 70-85% saturated ammonium sulfate increased their activity values by 3-fold, 6-fold, and 2.5-fold, respectively. However, partial purification of a novel protease from *B. licheniformis* strain K7A by $(NH4)_2SO_4$ fractionation (40-70%) plus dialysis increased the enzyme activity by 12-fold **(Hadjidj et al., 2018).**

The activity of the dialyzed enzyme was strongly inhibited in the presence of pepstatin (0.1 mM), iodoacetamide (10 mM), EDTA (10 mM) and PMSF (10 mM). The results of the inhibition study suggested that the enzyme preparation could contain all the four types of protease classes (Aspartic, Cysteine, and Serine and Metallo-proteases). Further purification studies or analytical fractionation of the crude enzyme would be required to elucidate this point with certainty. However, the low residual activity observed in the presence of pepstatin A (27%) could indicate the existence of one (or more) aspartic proteinase(s) in the crude enzyme mixture. However, it is interesting to observe that the activity of a purified serine alkaline protease from *B. mojavensis* was completely inhibited by more than one protease inhibitor agents; specifically PMSF (1mM) and iodoacetic acid (1mM) **(Beg and Gupta, 2003).** In contrast, the activities of milk-clotting protease from *B. licheniformis* USC13 **(Ageitos et al., 2007)** and novel protease from *B. licheniformis* K7A **(Hadjidj *et al.*, 2018)**

were completely inhibited only by PMSF. In other studies, the milk-clotting activities of purified milk-clotting aspartic protease from Withania coagulants fruit were exclusively suppressed by pepstatin A (0.08mM) **(Salehia *et al*., 2017).**

In the present study, the dialyzed enzyme from *B. subtilis* SMDFS-2B showed maximum activity at 55 ^{o}C. Similar to the present work, the purified milk-clotting enzyme from *B. sphaericus* **(El-Bendary *et al*., 2007)** and *B. subtilis* natto **(Wu *et al*., 2013)** showed optimal milk-clotting activity at 55 ^{o}C and 60 ^{o}C, respectively. However, milk-clotting activity from *B. licheniformis* 5A5 **(Ahmed and Helmy, 2012)** and *B. amyloliquefaciens* JNU002 **(Ding et al., 2012)** that showed maximum activity at 75 ^{o}C and 70 ^{o}C, respectively, are more thermo-tolerant than the proteinase from *B. subtilis* SMDFS-2B (this study). Different from the present study, the highest milk-clotting activity for milk-clotting protease from *B. licheniformis* USC13 (Ageitos et al., 2007) and purified enzyme from *B. subtilis* MTCC 10422 **(Kumari et al., 2016)** were obtained at 37 ^{o}C and 45 ^{o}C, respectively.

The thermal stability of the rennet enzyme was one of the most important criteria with respect to its potential applications. In this study, the result of the enzyme preparation from *B. subtilis* SMDFS-2B showed significant thermal stability. However, the MCA of the dialyzed enzyme was considerably reduced upon exposure at 60 ^{o}C. The enzyme retained 55% of its original activity after heating at 60 ^{o}C for 15 min; further incubation of the enzyme at 60 ^{o}C for 30 min caused a dramatic loss of its activity to 35%. The loss of milk-clotting activity upon increasing the reaction temperature may be interpreted to be due to the denaturation of the enzyme above the verified temperature **(Wehaidya *et al*., 2016)** and this maybe a technological advantage since no bitterness development can occur during cheese ripening due to the inactivated proteolytic action of the enzyme after the cooking process of the curd **(Sousa *et al*., 2001).**

The residual activity obtained for bacterial rennet from *B. licheniformis* 5A5 (73.4%) upon exposure for 1 h at 40 ^{o}C (Ahmed and Helmy, 2012), MCE from *B. subtilis* (81.3%) after incubation for 10 min at 55 ^{o}C (Ding *et al*., 2011), enzyme from *B. amyloliquefaciens* JNU002 (52.84%) after incubating for 20 min at 50 ^{o}C **(Ding et al., 2012)**, enzyme from *B. sphaericus* (30%) after 20 min incubation at 60 ^{o}C **(El-Bendary *et al*., 2007),** enzyme from *B. subtilis* natto (47%) after incubation for 40 min at 55 ^{o}C **(Shieh *et al*., 2009)** and enzyme from *B. subtilis* natto (50%) after incubation at 60 ^{o}C for 20 min **(Wu *et al*., 2013)** were comparable with the present study.

The pH had a significant effect on the activity of the milk-clotting enzyme. The dialyzed enzyme obtained from *B. subtilis* SMDFS-2B showed the highest milk-clotting activity at pH 5.5. The result from the current study inferred that the enzyme works best at acidic pH than alkaline pH. Similar to the present study, the maximal activity for milk-clotting enzyme from *B. subtilis* **(Wehaidya et al., 2016),** *B. subtilis* B1**(Ding et al., 2011),** *B. amyloliquefaciens* **D4 (He et al., 2011)**, *B. amyloliquefaciens* JNU002 **(Ding et al., 2012)**, *B. subtilis* natto **(Wu *et al*., 2013)** and *B. subtilis* MTCC 10422 **(Kumari et al., 2016)** were shown at pH 5.0, 5.5, and 6.0, respectively. On the other hand, the highest activities for milk-clotting protease from *B. licheniformis* strain USC13 **(Ageitos et al., 2007)** and milk-clotting enzyme from *B. sphaericus* **(El-Bendary *et al*., 2007)** were obtained at pH 7.5 and 6.0-7.5, respectively.

The enzyme showed a narrow range of pH stability after incubation at different pH at room temperature. The dialyzed enzyme retained 50-80% of its milk-clotting activity upon incubation at pH 5.0-6.5 for 1.0 h. However, any change of pH at either end of these ranges caused a significant reduction in residual milk-clotting activity. These findings are in accordance with values reported for purified milk-clotting enzymes from *B. amyloliquefaciens* JNU002 **(Ding et al., 2012)**, *B. amyloliquefaciens* D4 **(He et al., 2011),** *B. subtilis* natto **(Shieh *et al*., 2009)** and *B. subtilis* natto **(Wu *et al*., 2013)**. In another study, the milk-clotting enzyme from *B. subtilis* MTCC 10422 was shown stability in both acidic and basic pH **(Kumari et al., 2016).**

The enzyme showed an increment in its MCA with increasing skim-milk concentration. The highest milk-clotting activity was achieved at 20% skim-milk concentration. However, the maximal enzyme activity for milk-clotting enzyme (MCE) from *B. subtilis* **(Wehaidya et al., 2016)**, *B. amyloliquefaciens* D4 **(He et al., 2011),** *B. licheniformis* 5A5 **(Ahmed and Helmy, 2012)** and *B. sphaericus* **(El-Bendary *et al*., 2007)** were attained at 0.4%, 5%, 6% and 9% substrate concentration, respectively. The differences between the present finding and others could be inferred to the impurity and handling of the crude enzyme used in this study.

The effect of several metal ions on the MCA of the dialyzed enzyme was determined. The MCE was activated by most of the metal ions tested except Ni^{2+}, Cu^{2+}, and Co^{2+}. The activity of MCE extremely stimulated by $MnSO_4$, $FeSO_4$, $MnCl_2$, $MgCl_2$, and $CaCl_2$. Similarly, MCE from *B. licheniformis* 5A5 **(El-Bendary *et al*., 2007)**, *B. subtilis* **(Ding et al., 2011)**, *B. amyloliquefaciens* D4 **(He et al., 2011)** and *B. amyloliquefaciens* JNU002 **(Ding et al., 2012)** were also strongly triggered by Mn^{2+}, Ca^{2+}, Ca^{2+}, and Ca^{2+}, respectively. In other studies, Mg^{2+} and Mn^{2+} also showed a stimulatory effect on the MCA of MCE from *B. subtilis* MTCC 10422 **(Kumari et al., 2016).** Whereas, Ni^{2+}, Cu^{2+}, and Co^{2+} inhibited the MCA of MCE from *B. sphaericus* (Ding *et al*., 2012; El-Bendary *et al*., 2007). Likewise, Co^{2+} was found to have an inhibitory effect on the MCA of MCE from *B. subtilis* MTCC 10422 **(Kumari et al., 2016).**

Calcium had a positive effect on the activity of the MCE. It has been described as important in milk clot formation when its concentration is high enough **(He et al., 2011).** It is known that Ca^{2+} combines with ρ-casein to form a firm clot during the second phase of the clotting process. The addition of $CaCl_2$ to milk causes a reduction in milk-clotting time by rennet, this increases the rate of coagulation **(Ahmed and Helmy, 2012).** In the present study, the highest MCA of the dialyzed enzyme was obtained at 10 mM and 50 mM concentration of $MnSO_4$ and $CaCl_2$, respectively. Similar to this study, the maximum activity for MCE from *B. subtilis* B1 **(Ding et al., 2011)** and *B. amyloliquefaciens* JNU002 **(Ding et al., 2012)** were obtained at 50 mM and 60 mM $CaCl_2$ concentration respectively. On the other hand, the optimal milk-clotting activity for MCE from *B. licheniformis* 5A5 **(Ahmed and Helmy, 2012)** and *B. amyloliquefaciens* D4 **(He et al., 2011)** were detected at 10 mM and 25 mM $CaCl_2$ concentration respectively.

CONCLUSION

In the present study, the milk-clotting enzyme produced from *B. subtilis* SMDFS-2B by submerged fermentation revealed attractive properties. The partial characterization results have been shown that the enzyme exhibited optimum activity at 55 ^{o}C and pH 5.5. Further studies on the purification and full characterization of the enzyme(s) present in the crude preparation are in preparation.

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