Biocatalysis and Agricultural Biotechnology 21 (2019) 101356

Contents lists available at ScienceDirect

Biocatalysis and Agricultural Biotechnology

journal homepage: http://www.elsevier.com/locate/bab

Statistical optimization and biochemical characterization of bioactive *Bacillus megaterium* 314 caseinase produced on egg shell and molokihya stalks

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ARTICLE INFO

Keywords:
Egg shell
Molokihya stalk
Pretreatment
RSM
Thermodynamics
Biological activities

ABSTRACT

Egg shell (ES) was a good inducer for caseinase enzyme production by *Bacillus megaterium* 314 without adding supplements (only distilled water). Mixture of ES with molokihya stalk (MS) was the most suitable substrate that increased enzyme production by 4.7-fold. Optimized HCl pretreatment of ES using Central composite design enhanced enzyme production by 3.3-fold. Moreover, caseinase production was enhanced by 2.5-fold using Plackett-Burman (PB) followed by Central composite (CC) design. Enzyme showed maximum activity at temperature 50 °C and pH 9.0. Activation energy (*Ea*) and activation energy for the thermal denaturation (*Ed*) were 11.2 and 135.9 kJ/mol, respectively. Caseinase was highly tolerant to heat inactivation retained 93.6% of its initial activity after 1 h heating at 50 °C. In addition, the calculated half-life time ($t_{0.5}$) and decimal reduction time (*D*-value) at 50 °C were 26.9 and 89.3 h, respectively. Moreover, thermodynamic parameters (enthalpy, Gibbs free energy and entropy) indicated that the enzyme was highly tolerant to heat inactivation. Sesame protein was the most suitable for hydrolysis by caseinase. Optimization of Sesame proteolysis using CC design causing 16.9-fold increases compared with the unoptimized. *In vitro* results showed promising biological activities as anticoagulation and fibrinolytic for caseinase preparation.

1. Introduction

The cost of the production media can greatly affect the total production economics of microbial enzymes. The main goal of the research was the use of low cost substrate as agricultural and industrial wastes (AIW) for the production of industrially potent enzyme. Bioconversion of AIW into usable product is important for waste management, economic development and reduction of pollution problems (Nagamalli et al., 2017). Recently, efforts have been made to explore new resources to reduce the enzyme production cost by improving productivity, and using low-cost raw materials as substrate (Al-Abdalall and Al-Khaldi, 2016).

Egg shell (ES) is considered an animal by-product which is available in a large amount from the food processing (Verma et al., 2012). According to FAO (2016), the egg sector has increased rapidly, and now the production reaches 6.5×10^7 ton/year. Most of the ES waste is disposed without any prior treatment in landfills because it is traditionally worthless and finally causes serious environmental problems (unpleasant odors, flies and pathogenic agents) (Ho et al., 2013; Soares et al., 2013). ES is mainly composed of $CaCO_3$ (94%), organic substances (4%), $Ca_3(PO_4)_2$ (1%), $MgCO_3$ (1%) and smaller quantities from Ca, Mg, Si, Zn, etc. (Mittal et al., 2016). Thus, its reuse is an important method for decreasing environmental pollution as well as producing a useful product (King'ori, 2011; Intharapat et al., 2013). Applications of ES are limited due to its insolubility (in water and organic solvents) mainly due to a high number of disulfide bonds (Cheng et al., 2009). Therefore, suitable treatment is wanted to recover valuable Ca^{+2} from ES waste. Heat treatment of ES can be used to produce cheap and environmentally friendly catalysts.

AIW are low cost substrates rich in organic ingredients, which are essential sources of carbon and nitrogen and can be utilized by microorganisms. Molokihya stalks are agro waste from the *Corchorus olitorius* plant, cheap and profusely available in Egypt. In addition, molokihya is rich in protein, vitamins (vitamins A, B … etc) and minerals (Fe^{+2}, Ca^{+2}, Na^{+2} … etc).

Bioactive peptides are peptides having a biological activity (anticoagulation and fibrinolytic activities) at physiological scale that benefits the health of the body. It can be obtained *in vitro* through the

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https://doi.org/10.1016/j.bcab.2019.101356
Received 14 May 2019; Received in revised form 30 August 2019; Accepted 23 September 2019
Available online 24 September 2019

Table 1
Central composite design for pretreatment of egg shells with HCl for caseinase production.

Trail number	Factor 1		Factor 2		Factor 3	
	A: E.S weight (g)		B: HCl conc. (%)		C: pretreatment time (min.)	
	coded	actual	coded	actual	coded	actual
1	0.000	1.60	0.000	10.50	1.682	180.00
2	0.000	1.60	0.000	10.50	0.000	105.00
3	0.000	1.60	0.000	10.50	0.000	105.00
4	1.000	2.43	−1.000	4.85	−1.000	60.40
5	−1.000	0.77	1.000	16.15	−1.000	60.40
6	0.000	1.60	0.000	10.50	0.000	105.00
7	−1.682	0.20	0.000	10.50	0.000	105.00
8	0.000	1.60	0.000	10.50	−1.682	30.00
9	1.000	2.43	−1.000	4.85	1.000	149.60
10	−1.000	0.77	−1.000	4.85	1.000	149.60
11	1.000	2.43	1.000	16.15	−1.000	60.40
12	1.000	2.43	1.000	16.15	1.000	149.60
13	−1.000	0.77	−1.000	4.85	−1.000	60.40
14	0.000	1.60	1.682	20.00	0.000	105.00
15	1.682	3.00	0.000	10.50	0.000	105.00
16	−1.000	0.77	1.000	16.15	1.000	149.60
17	0.000	1.60	−1.682	1.00	0.000	105.00
18	0.000	1.60	0.000	10.50	0.000	105.00
19	0.000	1.60	0.000	10.50	0.000	105.00
20	0.000	1.60	0.000	10.50	0.000	105.00

Table 2
Effect of utilization of, a: extract of pretreated egg shells only + molokihya stalks, b: pretreated egg shells only + molokihya stalks, c: extract of pretreated egg shells + pretreated egg shells + molokihya stalks on *B.megaterium* 314 caseinase production.

Trail number	extract of egg shells only caseinase activity (U/ml) (a)	pretreated egg shells only caseinase activity (U/ml) (b)	extract + pretreated egg shells caseinase activity (U/ml) (C)
1	436.84	521.54	752.96
2	152.39	570.10	1013.60
3	1600.06	531.09	1013.60
4	1108.62	582.46	2896.00
5	1706.74	417.68	955.68
6	688.28	515.93	1013.60
7	1852.77	620.91	0.00
8	1385.45	440.98	839.84
9	0.00	342.74	3185.60
10	1522.60	405.61	318.56
11	1662.29	567.58	810.88
12	1509.90	704.56	781.92
13	1917.54	465.96	234.58
14	1930.24	627.93	234.58
15	516.85	812.35	1940.32
16	1344.82	449.96	0.00
17	1563.24	607.44	1766.56
18	1892.14	683.51	1013.60
19	2247.71	522.67	1013.60
20	2014.05	665.26	1013.60

enzymes produced by proteolytic organisms (Espeche Turbay et al., 2012). Inhibition of blood coagulation by anticoagulants can be used as an important way to prevent clot formation (Zong et al., 2017).

Statistical methods are used to improve the process by generating experimental models (Khusro, 2015). Response surface method (RSM) is a collection of mathematical and statistical models that help to identify the effective variables, its interactions saving both effort and time (Sreedevi et al., 2017).

Kinetic and thermodynamic properties supply useful information for enzyme industrial application about the enzyme activity and thermo stability (Souza et al., 2015).

The objective of this study was to estimate the feasibility of utilizing ES for the production of caseinase by *B. megaterium* 314. Utilization of AIW with ES for caseinase production. Optimization of ES pretreatment with HCl by CC design. Enhancement of *B. megaterium* 314 caseinase production using PB followed by CC design. Kinetic and thermodynamic characterization of caseinase enzyme. Application of produced caseinase for hydrolysis of different substances and biological activities (anticoagulation and fibrinolytic). Finally, we use CC design to optimize the hydrolysis of Sesame protein.

2. Materials and methods

2.1. Bacterial strain

The bacterial strain used in the present study *Bacillus megaterium* 314 was obtained from the Culture Collection of the National Research Centre, Egypt. It was maintained at 4 °C and grown on nutrient agar for 24 h at 37 °C before performing each experiment.

2.2. Raw materials preparation

Agricultural and industrial wastes (AIW) as corn cob (CC), potato shell (PS), pomegranate peel (PP), orange peel (OP), molokihya stalk (MS), rice straw (RS) and lemon peel (LP) were collected from local market in Egypt. They were washed to remove unwanted dust particles with distilled water and were dried at 50 °C in an oven overnight. Hen egg shells (ES) were collected, washed both outside and inside to get rid of dirt and other organic materials, dried (at 50 °C for 24 h) and crushed to small pieces (~0.25 cm). The dried wastes samples were ground, separated by 1 cm sieve and were packed in air-tight containers for use in the production medium.

2.3. Utilization of AIW for enzyme production

It was carried out in 250-ml Erlenmeyer flask contained 2 g of ES in 50 ml of distilled water. AIW (CC, PS, PP, OP, MS, RS, and LP) were tested individually and with ES in a ratio 1:1 to evaluate their induction for caseinase production. After sterilization, each flask was inoculated with 1 ml of cell suspension (1 slant 24 h-old in 20 ml H_2O) with OD~0.4 at 600 nm. Then the flasks were incubated in a rotary shaker at 150 rpm and 35 °C for 48 h.

2.4. Central composite design for ES pretreatment with HCl

Pretreatment of ES waste was carried out by using HCl according to Vasan et al. (2013) method. After treatment, the extracts were kept in flasks and the residues (pretreated ES) were collected and washed extensively with H_2O until neutral pH was reached, filtered and dried. We studied the quantitative effect of three pretreatment variables namely (A) ES weight (g), (B) HCl concentration (%) and (C) time of pretreatment (min) on caseinase production. Each variable was studied with five levels −1.682, −1, 0, +1 and + 1.682 which gave 20 trial as shown in Table 1. The effect of utilization of, a: extract of pretreated ES only + molokihya stalks, b: pretreated ES only + molokihya stalks, c: extract of pretreated ES + pretreated ES + molokihya stalks on caseinase production (Table 2). Statistical analysis of the model was performed using the analysis of variance (ANOVA) (Table 3).

2.5. Optimization of alkaline protease production by statistical factorial design

2.5.1. Plackett-Burman design

In this design we investigated the effect of eleven factors on caseinase production including A: incubation time (h), B: shaking rate (rpm), C: pH, D: inoculum size (ml), E: Glucose (g/l), F: lactose (g/l), G: casein (g/l), H: $(NH_4)_2SO_4$ (g/l), J: NaCl (g/l), K: BaCl (g/l), L: molokihya stalk weight (g/flask).

Each of these factors was studied with low level (−1) and high level (+1). Total number of experiments was 12 trial based on the rule n+1,

Table 3
Analysis of variance (ANOVA) for CCD for pretreatment of egg shells with HCl for caseinase production.

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F	
Model	51499.05	6	8583.174187	65.37	<0.0001	significant
A-E.S weight	25428.59	1	25428.58723	193.66	<0.0001	
B–HCl conc.	12697.39	1	12697.39216	96.70	<0.0001	
C-pretreatment time	163.98	1	163.9820615	1.25	0.2840	
AB	11682.25	1	11682.24909	88.97	<0.0001	
AC	626.07	1	626.0668051	4.77	0.0479	
BC	900.77	1	900.7677901	6.86	0.0212	
Residual	1706.97	13	131.3057346			
Lack of Fit	1706.97	8	213.3718188			
Pure Error	0.00	5	0			
Cor Total	53206.02	19				

R^2 0.9679, Adj R^2 0.9531, Pred R^2 0.8445, Adeq Precision 29.564, C.V. % 17.63.

Table 4
Plackett- Burman design for *B.megaterium* 314 caseinase production.

Trial number	A:incubation time (h)	B:shaking (rpm)	C: pH	D:inoculum size (ml)	E:glucose (g/l)	F:lactose (g/l)	G: casein (g/l)	H: $(NH_4)_2SO_4$ (g/l)	J:NaCl (g/l)	K:BaCl (g/l)	L:molokihya weight (g/ flask)	caseinase activity (U/ ml)
1	48.00	100.00	9.00	2.00	1.00	0.00	0.00	0.00	1.00	0.00	2.00	2200.54
2	24.00	180.00	7.00	2.00	1.00	0.00	1.00	1.00	1.00	0.00	1.00	5144.31
3	48.00	100.00	9.00	2.00	0.00	1.00	1.00	1.00	0.00	0.00	1.00	4173.38
4	24.00	100.00	7.00	2.00	0.00	1.00	1.00	0.00	1.00	1.00	2.00	1688.66
5	48.00	180.00	7.00	1.00	0.00	1.00	0.00	1.00	1.00	0.00	2.00	5668.94
6	24.00	180.00	9.00	2.00	0.00	0.00	0.00	1.00	0.00	1.00	2.00	4022.18
7	48.00	100.00	7.00	1.00	1.00	0.00	1.00	1.00	0.00	1.00	2.00	1386.27
8	48.00	180.00	9.00	1.00	0.00	0.00	1.00	0.00	1.00	1.00	1.00	5652.55
9	24.00	180.00	9.00	1.00	1.00	1.00	1.00	0.00	0.00	0.00	2.00	3907.42
10	48.00	180.00	7.00	2.00	1.00	1.00	0.00	0.00	0.00	1.00	1.00	4869.24
11	24.00	100.00	9.00	1.00	1.00	1.00	0.00	1.00	1.00	1.00	1.00	2861.80
12	24.00	100.00	7.00	1.00	0.00	0.00	0.00	0.00	0.00	0.00	1.00	3643.28

Table 5
Analysis of variance (ANOVA) for PBD for *B.megaterium* 314 caseinase production.

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F	
Model	23604306.94	8	2950538.37	42.94	0.0052	significant
A-incubation time	599996.97	1	599996.97	8.73	0.0598	
B-shaking	14764603.13	1	14764603.13	214.89	0.0007	
E-glucose	1672093.74	1	1672093.74	24.34	0.0160	
G-casein	143752.07	1	143752.07	2.09	0.2438	
H-$(NH_4)_2SO_4$	139792.14	1	139792.14	2.03	0.2490	
J-NaCl	123025.53	1	123025.53	1.79	0.2732	
K–BaCl	1510292.23	1	1510292.23	21.98	0.0183	
L-molokhia weight	4650751.14	1	4650751.14	67.69	0.0038	
Residual	206122.49	3	68707.50			
Cor Total	23810429.43	11				

R^2 0.9913, Adj R^2 0.9683, Pred R^2 0.8615, Adeq Precision 18.488, C.V. % 6.96.

where n: represents the number of factors under investigation. In the experimental design, each row represented an experiment, and each column represented an independent variable (Table 4). The statistical significance was determined by F-value, and the proportion of variance explained by the model obtained was given by the multiple coefficient of determination, R^2 (Table 5). Experimental responses were analyzed by first order model by the following equation: caseinase activity (U/ml) = $\beta^\circ + \Sigma\beta i Xi$.

β° is the model intercept and βi is the linear coefficient, and Xi is the level of the independent variable.

2.5.2. Central composite design

In this design we studied the quantitative effect of the two most effective factors determined from the Plackett-Burman design including (A) incubation time and (B) shaking rate.Variables were investigated with three levels −1, 0 and + 1 as shown in Table 6 which gave the total of 13 trial. Statistical analysis of the model was performed using the analysis of variance (ANOVA) (Table 7).

2.6. Enzyme partial purification

The culture of *Bacillus megaterium* 314 under optimized conditions was centrifuged. Ethanol was added to the supernatant to achieve 75% concentration. The mixture was stored at 4 °C for 10 min, and the precipitate was collected by centrifugation at 10,000×*g*, 20 min at 4 °C. The precipitate was dissolved in H_2O and both enzyme activity and protein content were determined.

2.7. Enzyme assay

Caseinase activity was assayed using the method of Asha and Palaniswamy (2018). The enzyme solution (0.25 ml) was mixed with 0.25 ml of casein solution (1.5% in 0.2 M glycin-NaOH pH 10) and incubated for 30 min at 40 °C. The reaction was stopped by adding 0.5 ml of

Table 6
Central composite design for *B.megaterium* 314 caseinase production.

Trial number	Factor (A): incubation time (days)		Factor (B): shaking rate (rpm)		caseinase activity (U/ml)	
	Coded	Actual	Coded	Actual	Actual	Predicated
1	1	7	1	200	6021.57	5961.76
2	0	4.5	0	150	6164.00	6136
3	0	4.5	0	150	6164.00	6136
4	0	4.5	0	150	6164.00	6136
5	0	4.5	0	150	6164.00	6136
6	1	7	0	150	5045.60	5187.04
7	0	4.5	1	200	6808.00	6856.16
8	0	4.5	0	150	6164.00	6136
9	−1	2	0	150	7221.77	7220.32
10	0	4.5	−1	100	5006.44	5098.24
11	1	7	−1	100	4176.40	4094.72
12	−1	2	−1	100	6247.20	6236.96
13	−1	2	1	200	7874.40	7886.08

trichloroacetic acid (10%), and was centrifuged at 10,000×*g* for 20 min. The amount of tyrosine released from the hydrolyzed proteins in the supernatant was measured according to the method of Lowry et al. (1951). All the experiments were carried in triplicate and the results expressed as mean values ± SD.

2.8. Protein determination

The protein content of the enzyme preparation was determined by the method of Lowry et al. (1951).

2.9. Biochemical characterization of caseinase enzyme

2.9.1. Effect of temperature on enzyme activity

The effect of temperature on caseinase activity was determined from 30 to 70 °C in 10 °C increments at pH 10 for 30 min.

The activation energy (*Ea*) was calculated from the slope of the Arrhenius plot (drawn between log of residual activity % versus absolute temperature in Kelvin X10^3) according to the following equation (Singh et al., 2010):

$$\text{Slope} = -\ Ea\ /\ 2.3\ \text{R} \quad (1)$$

Where: R is the gas constant (8.314 J/mol).

The temperature quotient value (Q10) is the rate of an enzymatic catalysis reaction changing for every 10 °C rise in temperature, was calculated by the equation of Ghosh et al. (2015):

$$\ln Q10 = (Ea \times 10)/RT^2 \quad (2)$$

where: T is the absolute temperature in Kelvin (°K).

2.9.2. Effect of pH on enzyme activity

The optimal pH for caseinase activity was determined within a pH range of 6–11. The partial purified enzyme (0.25 ml) was mixed with 0.25 ml of 0.2 M of sodium phosphate buffer (pH 6 and pH 8), and glycine-NaOH buffer (pH 9–11). The enzyme activity was measured by casein as substrate (Meshram et al., 2017).

2.9.3. Determination of kinetic constants of caseinase enzyme

Caseinase was assayed as described previously, the reaction mixture contained varying amounts of casein from 0.5 to 10% (w/v) in glycine-NaOH buffer (0.2 M and pH 9). The maximum reaction velocity (*Vmax*), Michaelis–Menten constant (*Km*), catalytic efficiency (*Vmax/Km*) were determined as described by Lineweaver–Burk plot method Lineweaver and Burk (1934) at optimum assay conditions.

2.9.4. Enzyme stability

2.9.4.1. Stability toward heat inhibition. Thermal stability of the enzyme was determined by measuring the residual activity (RA%) of the enzyme over temperature range of 50–70 °C for different times (from 0 to 60 min) as follows:

$$\text{Residual activity (RA\%)} = (\text{Observed activity/ Initial activity}) \times 100 \quad (3)$$

Deactivation rate constant (*kd*) was calculated from the Arrhenius plot of Log RA versus of time at the temperature used for inactivation was calculated as follows:

$$\text{Slope} = -\ kd \quad (4)$$

Half-lives ($t_{1/2}$) and decimal reduction time (*D*-value) were determined as follows:

$$t_{0.5} = \ln 2/\ kd \quad (5)$$

$$D\text{-value} = \ln 10/\ kd \quad (6)$$

The activation energy (*Ed*) for caseinase denaturation was determined from Arrhenius plot of (ln *kd*) as a function of (1/Temperature) in Kelvin (°K) using the following equation:

$$\text{Slope} = -Ed/\ \text{R} \quad [7]$$

The change in enthalpy (ΔH°), free energy (ΔG°) and entropy (ΔS°) for thermal inactivation of enzyme were determined as pointed by da Silva et al. (2018) as follows:

$$\Delta H^\circ = Ed - RT \quad (8)$$

$$\Delta G^\circ = -RT \ln (Kd.\ h/\ Kb.T) \quad (9)$$

$$\Delta S^\circ = (\Delta H^\circ - \Delta G^\circ)/\ T \quad (10)$$

Where: *Ed* = activation energy for denaturation (KJ/mol)), R = gas constant (8.314 J/mol/K), T = absolute temperature (°K), *kd* = deactivation rate constant (/min), h = Planck constant (11.04×10^{-36} J min) and *Kb* = Boltzman constant (1.38×10^{-23} J/K).

Table 7
Analysis of variance (ANOVA) for CCD for *B.megaterium* 314 caseinase production.

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F	
Model	10918657.92	5	2183731.58	338.52	<0.0001	significant
A-incubation time	6201264.80	1	6201264.80	961.31	<0.0001	
B-shaking	4635713.70	1	4635713.70	718.62	<0.0001	
AB	11877.49	1	11877.49	1.84	0.2169	
A^2	12649.80	1	12649.80	1.96	0.2041	
B^2	69635.06	1	69635.06	10.79	0.0134	
Residual	45155.74	7	6450.82			
Lack of Fit	45155.74	3	58.80			
Pure Error	0.00	4	0			
Cor Total	10963813.66	12				

R^2 0.9959, Adj R^2 0.9929, Pred R^2 0.9668, Adeq Precision 69.482, C.V. % 1.32.

Table 8
CCD for proteolysis of sesame by *B.megaterium* 314 caseinase.

Run	Factor A [caseinase units] U/ml	Factor B [sesame weight] gm	Factor C [Time] h	protein content mg/ml
1	2000.00	1.00	96.00	49.33
2	750.00	1.00	96.00	42.98
3	2000.00	2.50	30.00	19.33
4	750.00	1.00	30.00	5.95
5	1375.00	3.05	63.00	43.85
6	1375.00	1.75	120.16	64.35
7	1375.00	1.75	63.00	32.25
8	2000.00	2.50	96.00	64.89
9	1375.00	0.45	63.00	19.78
10	750.00	2.50	96.00	65.98
11	1375.00	1.75	5.84	0.00
12	1375.00	1.75	63.00	32.25
13	2000.00	1.00	30.00	11.25
14	1375.00	1.75	63.00	32.25
15	1375.00	1.75	63.00	32.25
16	2457.53	1.75	63.00	35.45
17	292.47	1.75	63.00	30.85
18	1375.00	1.75	63.00	32.25
19	750.00	2.50	30.00	20.25
20	1375.00	1.75	63.00	32.25

Table 9
Analysis of variance (ANOVA) for CCD for proteolysis of sesame by *B.megaterium* 314 caseinase.

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F	
Model	6345.30	6	1057.55	249.20	<0.0001	significant
A-A protease units	22.14	1	22.14	5.22	0.0398	
B–B sesame weight	752.33	1	752.33	177.28	<0.0001	
C–C Time	5514.70	1	5514.70	1299.47	<0.0001	
AB	23.32	1	23.32	5.49	0.0356	
AC	0.10	1	0.10	0.02	0.8825	
BC	32.72	1	32.72	7.71	0.0157	
Residual	55.17	13	4.24			
Lack of Fit	55.17	8	6.90			
Pure Error	0.00	5	0			
Cor Total	6400.47	19				

R^2 0.9914, Adj R-Squared 0.9874, Pred R-Squared 0.9587, Adeq Precision 56.412 C.V. % 6.17.

2.9.4.2. Stability in presence of metal ions and some additives. The activity of caseinase enzyme was investigated in the presence and absence of various metal ions and additives (0.01 M) after 1 h at 30 °C (Meshram et al., 2017). The activity of the enzyme in the absence of metal ions and additives was considered as 100% activity.

2.10. Applications of B. megaterium 314 caseinase

2.10.1. Evaluation of its proteolytic activity

2.10.1.1. Decomposition of ES and other protein substances. Decomposition of protein substances (cantaloupe, CC, ES, lentil, LP, OP, rice, RS, saw dust, sesame, wheat bran and PP) by *Bacillus megaterium* 314 caseinase was estimated by measuring the proteins released (Cheng et al., 2009). The reaction mixture consisted of 0.5 ml of glycine-NaOH buffer, 0.1 g of protein substances and 0.25 ml of enzyme solution (1974 U) at 30 °C for 24 and 48 h with shaking at 150 rpm. The sample was centrifuged at 4 °C and 10,000×*g* for 20 min. Protein concentrations in the supernatant were measured by the methods of Lowry et al. (1951).

2.10.1.2. Central composite design for optimization of proteolysis of sesame. In this design we studied the quantitative effect of the three effective factors including (A) caseinase units, (B) Sesame weight (g) and (C) incubation time.Variables were investigated with five levels −1.732, −1, 0, +1 and + 1.732 as shown in Table 8 which gave the total of 20 trial. Statistical analysis of the model was performed using the analysis of variance (ANOVA) (Table 9).

2.10.2. Biological activities

2.10.2.1. In vitro testing of anti-coagulation activity. To investigate the anticoagulant effect of samples preparations we evaluated the inhibition of blood clotting in presence of partial purified enzyme (1), production media (2) and crude enzyme (3). Anticoagulation activities of the samples preparations were determined according to the time required for clot formation and were compared with that of standard heparin sodium preparation (Colman et al., 1994; Ragab et al., 2018). Glass test tubes were cleaned by immersion for 24 h in chromic acid. To each tube, 0.8 ml of each sample (0.01% in saline), 0.8 ml saline solution (blank) or 0.8 ml of standard heparin sodium (control). One ml plasma and 0.2 ml of 1% $CaCl_2$ were added, respectively. Samples were incubated at 37 °C in water bath and the time was recorded immediately and each tube was closed. The time required for clot formation was then determined as an average of three readings. In addition, different dilutions from the positive sample preparations (10, 100, and 1000 times) were tested.

2.10.2.2. In vitro testing of fibrinolytic activity. Fibrinolytic activities were assayed with some modification according to the method of (USA, 1960; Ragab et al., 2018) as lysis percentages (%) of the plasma clot by the investigated samples. For comparison, a standard preparation of Hemoclar (Pentosan sulfuric polyester, product of Clin Midy. Paris) was used. Sets of 3 glass test tubes were cleaned by immersion overnight in chromic acid. To each tube 0.8 ml saline, 1 ml plasma and 0.2 ml $CaCl_2$ (1% w/v) were added. After mixing, the tubes were incubated in a water bath at 37 °C. When clotting was completed, 1 ml of either the saline solution, Hemoclar preparation (2 mg/ml), or the tested samples (partial purified enzyme (1), production media (2) and crude enzyme (3)) were added individually. The lysis percentages of the plasma clots were recorded with each sample and were compared with Hemoclar drug as standard.

3. Results and discussions

3.1. Utilization of AIW for caseinase production

The results in Fig. 1 indicated that the production of caseinase enzyme by *Bacillus megaterium* 314 was different according to the type of substrate and its combination with ES. ES was a good inducer for the production of caseinase producing 8.37 and 12.86 U/ml at concentration 1 and 2 g/50 ml, respectively. Our result was higher than Meshram et al. (2017) by 3.1 and 4.7 times, respectively. Mixing the molokihya stalk (MS) with the ES improved enzyme production by 4.71 and 4.05-fold compared to ES and MS individually, respectively. The results revealed that no activity was obtained when using only some wastes (CC, PP, OP and LP). The suitability of ES and MS wastes may be due to their constituents and sufficient nutrients. Jew's Mallow (English common name of molokihya) is an annual plant in the Middle East rich in mineral salt, protein and vitamins. Ahmed and Mostafa (2013) reported that dry samples of MS are rich in mineral salt (16.45%) such as iron, phosphorus, calcium, sodium, magnesium, potassium, etc. Moreover, MS contains protein (22.0%), pectic substance (21.20%), ash (18.0%), moisture (10.0%), fat, fiber and vitamins (A, B … etc).

On the other hand, MS is a cheap agro waste from the plant and abundantly available in Egypt. In addition, ES contains calcium carbonate, protein membrane, amino acid, collagen, keratin and mineral

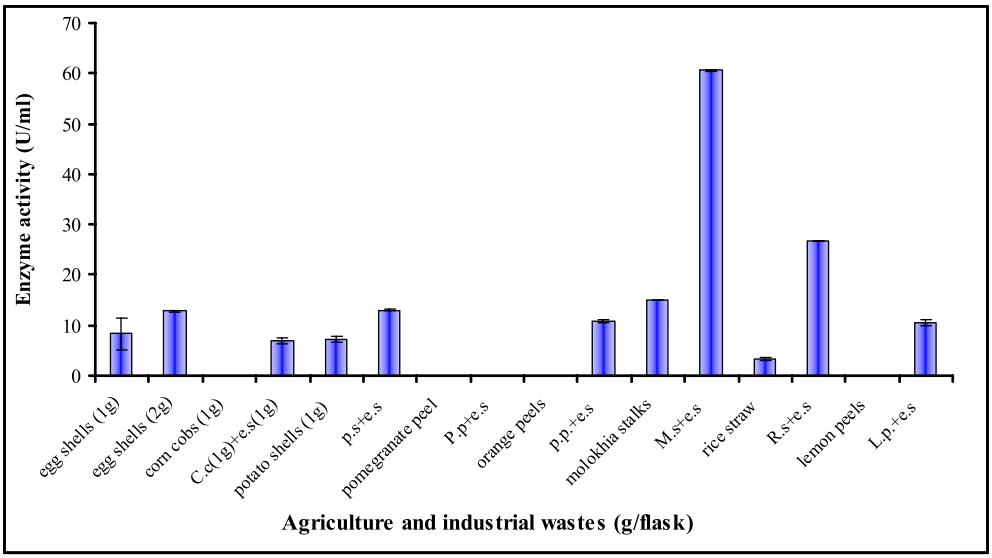

Fig. 1. Uitilization of different AIW for *B. megaterium* 314 caseinase production.

salts (Verma et al., 2012).

3.2. Improvement of caseinase production by ES pretreatment with HCl

Calcium is a key signaling molecule in all living organisms and is often trapped in intracellular compartments. In this step we tried to enhance the utilization of ES by HCl pretreatment for alkaline protease production. This can be attributed to that HCl pretreatment caused conversion of insoluble form $CaCO_3$ contained in ES (~95%) into soluble form $CaCl_2$ that microorganism can utilize (Verma et al., 2012).

As shown in Table 1 the HCl concentration, the ES weight and the pretreatment time significantly affected the yield percent. We studied the effect of utilization of, extract of pretreated ES with MS (a), pretreated ES extract with MS (b) and pretreated ES, pretreated ES extract with MS (c) on caseinase production as shown in Table 2. It could be noticed that the utilization of extract of pretreated ES only without ES (a) caused only 2.32-fold increase in trail 19 (2247.71 U/ml). While the utilization of pretreated ES only (b) did not enhance caseinase production on contrary it caused reduction in production. This can be due to the extraction of the majority of Ca^{2+} so the pretreated ES do not contain sufficient amount of Ca^{2+} to support caseinase production.

The highest caseinase activity (3185.60 U/ml) causing 3.28-fold increase was obtained in trial 9 by utilization of pretreated ES with its extract (c) after pretreatment of 2.43 g of ES with HCl 4.85% for 149.60 min. The enzyme activity (U/ml) can by calculated from the following equation:

Caseinase activity (U/ml) = +1039.95 + 690.41* ES weight −487.87 * HCl concentration – 55.44 * time of pretreatment −611.42 * ES weight * +141.54 * ES weight * time of pretreatment −169.78 * HCl concentration * time of pretreatment.

The analysis of variance as shown in Table 3 indicated the effectiveness of the design for the caseinase production. This indicated by the value of R^2 (0.9679) which meant that 96.79% of the results can be explained by the design (Montgomery, 1997). As the values of predicted R^2 of 0.9531 and adjusted R^2 0.8445 were closer to each other this meant the success of the model.

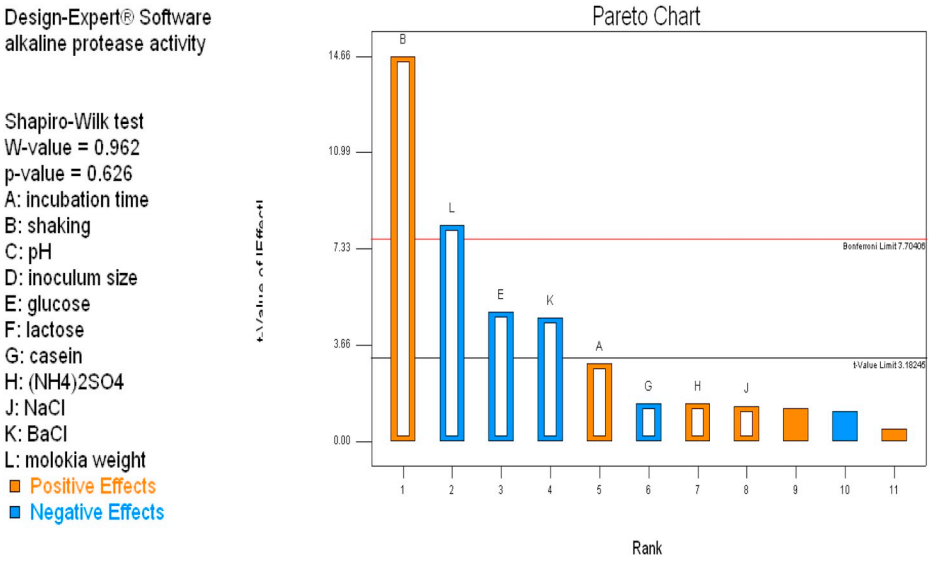

Fig. 2. Pareto chart showing the effect of each factor on *B.megaterium* caseinase production.

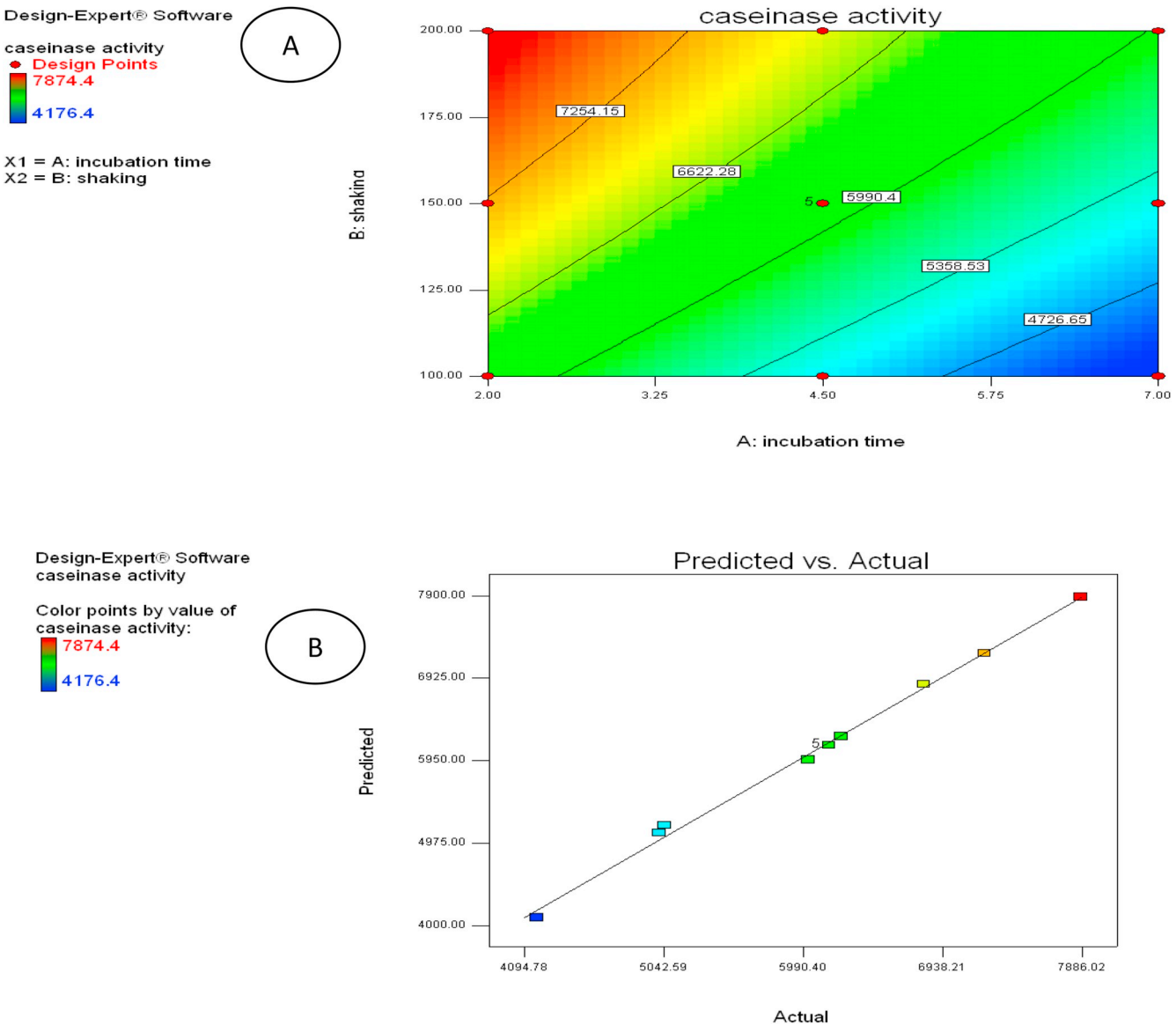

Fig. 3. (A) Contour plot showing interaction between the incubation time and the shaking rate and (B) Parity plot to show the distribution of observed and predicted values for *B.megaterium* caseinase production.

3.3. Optimization of caseinase production

In order to obtain high and commercially viable yields of caseinase it was essential to optimize fermentation media for the growth and production of enzyme.

3.3.1. Plackett-Burman design

We optimized and studied the qualitative effect of eleven factors as shown in Table 4 on *B. megaterium* 314 caseinase production. Caseinase activity (U/ml) can by calculated from the following equation:

Caseinase activity (U/ml) = +3768.21 + 223.61 * incubation time +1109.23 * shaking rate −373.28 * glucose −109.45 * casein +107.93 * $(NH_4)_2SO_4$ +101.25 * NaCl −354.76 * BaCl −622.55 * MS weight.

As shown in Pareto chart (Fig. 2) incubation time and shaking rate had the highest positive effect on caseinase production. On contory, glucose showed negative effect on caseinase production similar to that reported by Nagamalli et al. (2017). The addition of casein as organic nitrogen source and $(NH_4)_2SO_4$ as inorganic nitrogen source showed negative effect and positive effect, respectively on caseinase production in contrast to that reported by Nagamalli et al. (2017). The addition of some salts as NaCl and BaCl showed positive and negative effect, respectively. Al-Abdalall and Al-Khaldi (2016) reported the promotive effect of KNO_3 and NH_4Cl and inhibitory effect of $CoCl_2$ on *B. subtilis* alkaline protease production. While lactose, pH and inoculums size did not show significant effect on *B.megaterium* 314 caseinase production.

As shown in Table 4 the highest caseinase production was obtained in trial number 5 (5668.94 U/ml) causing 1.78-fold increase in enzyme production if compared with unoptimized medium.

The effectiveness of the design for caseinase production was indicated by analysis of variance as shown in Table 5. The success of the design was confirmed by the value of R^2 0.9913 indicating that 99.13% of the variability in the response could be explained by the model.

3.3.2. Central composite design

The interaction between incubation time and shaking rate was illustrated by counter plot (Fig. 3A). As Shown in Table 6 the highest caseinase production 7874.40 U/ml was achieved in trial number 13 causing 1.39 and 2.47-fold increase if compared with the previous step of optimization and unoptimized medium, respectively. The highest

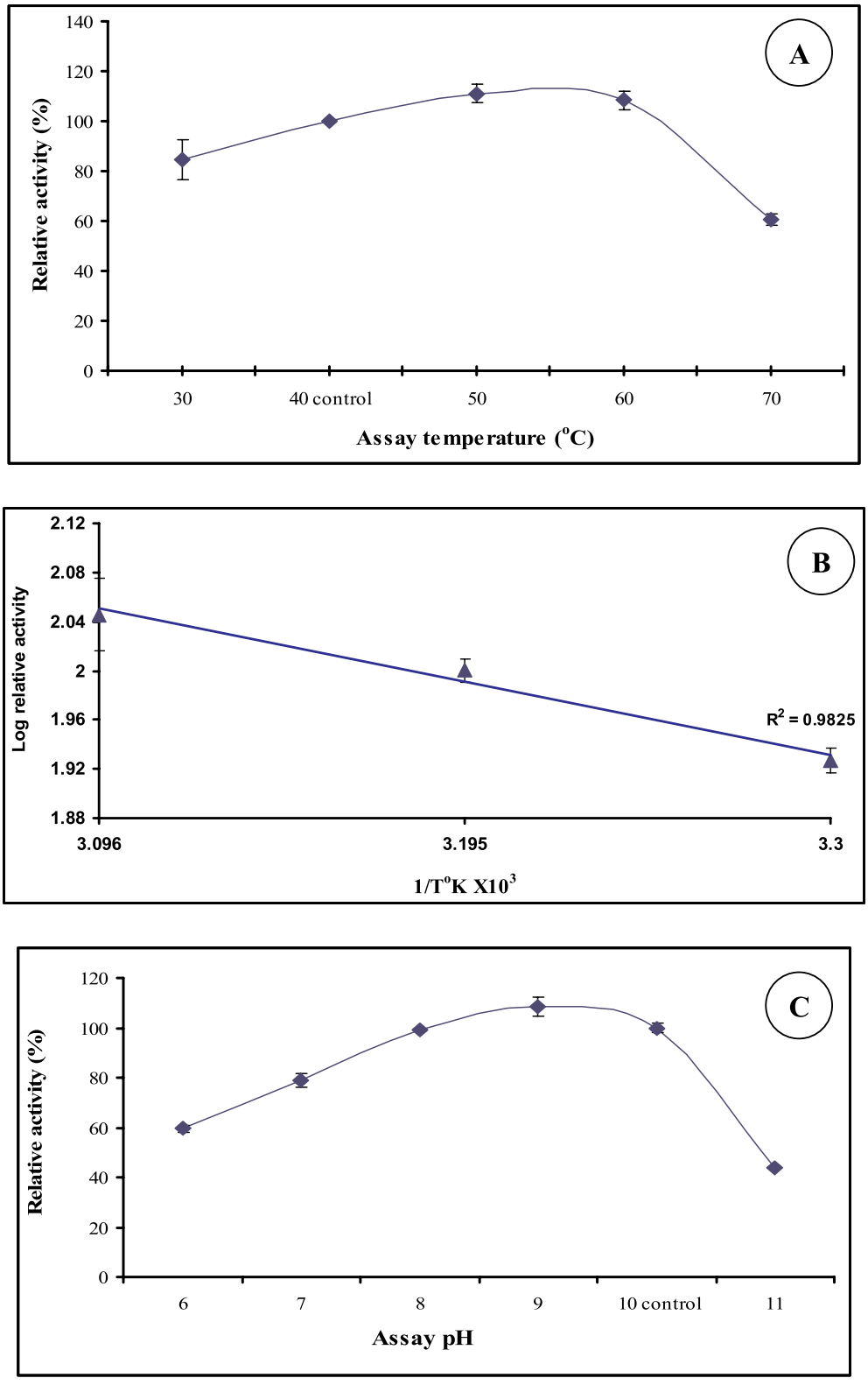

Fig. 4. Effect of temperature (A), Arrhenius plot for activation energy (B) and pH (C). on the activity of *B.megaterium* 314 caseinase.

caseinase production was obtained after 2 days as reported by Nagamalli et al. (2017). Caseinase activity (U/ml) can by calculated from the following equation:

Caseinase activity (U/ml) = +6136.00–1016.63 * incubation time +878.99 * shaking rate +54.49 * incubation time * shaking rate +67.68 * incubation time^2 -158.79 * shaking rate^2

Observed and predicted caseinase activity was plotted in Fig. 3B and it suggested a satisfactory correlation between experimental and predicted value, where the data points localized close to the diagonal line. The goodness of fit of the model was checked by determination of coefficient R^2 0.9959 the closer the R^2 is to1.0, the stronger the model and the better it predicts the caseinase production. The Predicted R^2 value of

Table 10
Kinetic and thermal properties of *B. megaterium* 314 caseinase.

Parameters	Value
Optimum temperature (°C)	50
Activation energy (*Ea*) (KJ/mol)	11.20
Vmax (U/mg protein)	4000
Km (mg casein/ml)	1.67
Vmax/Km (U/mg protein/mg casein/ml)	2395
Activation energy for denaturation E_d (KJ/mol)	135.9
Temperature quotient Q10	1.0

0.9668 was in reasonable agreement with the adjusted R^2 value of 0.9929. The model F-value of 338.52 implied the model was significant (Table 7). Values of Prob > F less than 0.0500 indicated model terms were significant. Low CV% value 1.32 predicted accuracy and reliability of the experiments conducted (Sen and Swaminathan, 2004).

3.4. Biochemical characterization of caseinase enzyme

For industrial applications, proteases must possess activity and stability under relatively harsh conditions, often including extremes in pH, temperature and presence of oxidizing agents and inhibitors. The partially purified caseinase with enzymatic activity (7417.2U/ml) and specific activity (1456.3U/mg protein) will be used in the following experiments.

3.4.1. Effect of temperature on enzyme activity

As illustrated in Fig. 4 A at 50 °C, enzyme showed maximum value of 111.11% relative activity. The enzyme activity reduced above 50 till 60 °C, however at 70 °C, the enzyme lost 40% of its activity. In general, for detergent applications the proteases should be active at temperature more than 40–50 °C as pointed by Mothe and Sultanpuram (2016).

Arrhenius plots for temperature data (Fig. 4B, Table 10) of caseinase enzyme *Ea* was 11.20 kJ/mol. Low *Ea* indicated that the enzyme has massive catalytic efficiency, due to low energy required to make the activated complex of caseinase-casein.

As presented in Table 10, the Q_{10} value for caseinase at different temperatures was 1.0. Deviation from Q_{10} values means that the catalytic reactions do not depend on temperature and are controlled by other factors Singh et al. (2010).

3.4.2. Effect of pH on enzyme activity

The enzyme exhibited high activity in the pH range 8–11 with maximum activity at pH 9. As illustrated in Fig. 4C the enzyme showed low activity at pH 6 and 11 by 40.5 and 55.9%, respectively.

3.4.3. Kinetic constants of the caseinase enzyme

The highest specific activity of caseinase (3088.4 U/mg protein) was obtained at 5% casein concentration. The *Km* and *Vmax* of caseinase enzyme were 1.67 mg casein/ml and 4000 U/mg protein, respectively (Fig. 5, Table 10). The *Km* value gives an idea about the affinity of an enzyme to its substrate Sinha and Khare (2015). Low *Km* of enzyme indicates the high affinity between substrate and enzyme active site and consequently high enzyme activity. The catalytic efficiency ratio (*Vmax/Km*) was calculated to be 2395 U/mg protein/mg casein/ml which was taken as the criterion to estimate the substrates specificity (Castro et al., 2015).

3.4.4. Enzyme stability

3.4.4.1. Stability towards heat inhibition. Thermal inactivation of caseinase enzyme was studied in the range of 40–70 °C (Fig. 6A) and the inactivation parameters were summarized in Table 11. The enzyme was highly tolerant to heat inactivation retaining 93.6% of its initial activity after heating at 50 °C for 60 min.

As shown in Fig. 6B caseinase deactivation rate constant (*kd*) increased gradually with temperature, which meant that its irreversible denaturation had become clear. For example, the *kd* at 60 °C and 70 °C were higher than that at 50 °C by 7.6 and 73.5-fold, respectively.

As well known, $t_{0.5}$ is the time needed to reduce enzyme activity by 50% of the initial value at a given temperature. It is an important economic factor in many industrial processes where increasing $t_{0.5}$ means increasing enzyme thermostability. Calculated $t_{0.5}$ at 50 and 60 °C were 26.87 and 3.54 h, respectively. These values were longer than those reported by da Silva et al. (2018) at the same temperature (1.61 and 0.66 h, respectively).

Decimal reduction time of enzyme (*D*-value) is defined as the time, needed to reduce the initial activity to 10%. As recorded in Table 11 caseinase enzyme showed good stability at 50 °C, because exposition for more than 89 h was necessary to reduce its initial activity to 10%. It was massive results which was 22.31-fold higher than that reported by da Silva et al. (2018).

Activation energy for thermal denaturation of caseinase (*Ed*) 135.9 KJ/mol which was higher than that reported for an alkaline protease by da Silva et al. (2018).

The enthalpy (ΔH°) is an important thermodynamic parameter that

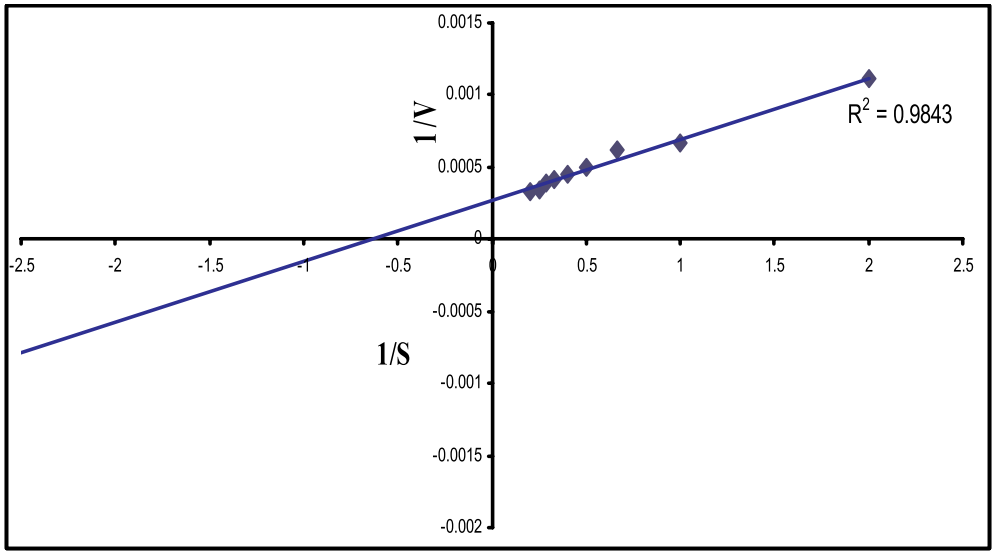

Fig. 5. Lineweaver–Burk plot of *B.megaterium* 314 caseinase.

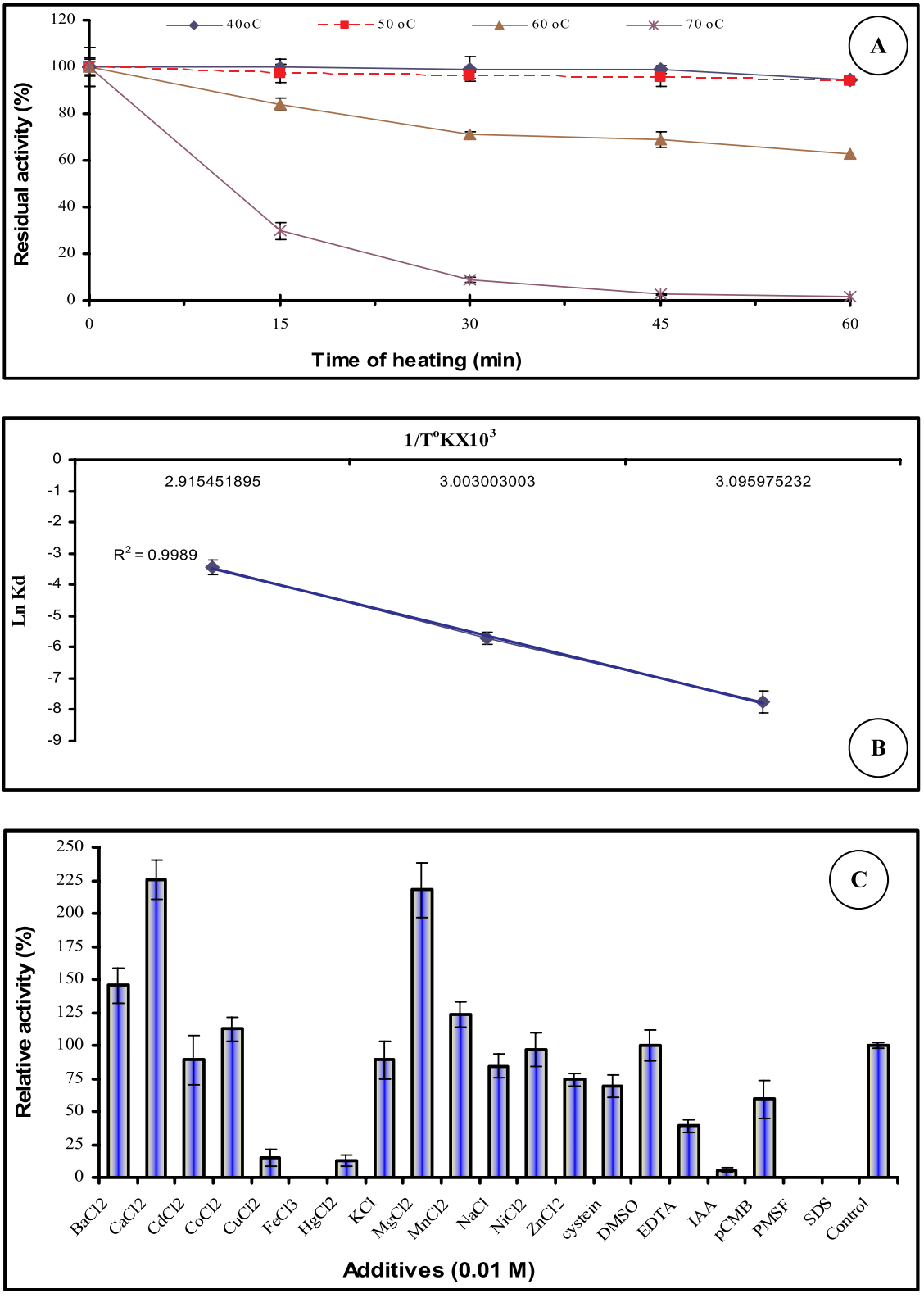

Fig. 6. Stability of *B.megaterium* 314 caseinase to heat treatment (A), Arrhenius plot for activation energy of denaturation (B) and in presence of metal ions and additives (C).

reflects the total amount of energy needed to destroy the enzyme. So, large or positive value of ΔH° is associated with high stability of enzyme to heat inactivation (Souza et al., 2015; da Silva et al., 2018). Table 11 indicated that decrease in ΔH° values was accompanied by an increase in temperature.

Gibbs free energy (ΔG°) is another thermodynamic parameters and good indicator of the enzyme stability Souza et al. (2015). An increase in ΔG° reveals an increase in enzyme thermostability on the contrary, a smaller or negative value is associated with a more spontaneous process and the enzyme becomes less stable da Silva et al. (2018). As shown in

Table 11
Thermodynamic parameters of thermal denaturation *B. megaterium* 314 caseinase.

Parameters	Temperature (°C)		
	50	60	70
Denaturation rate constant (K_d):/min	0.4×10^{-3}	3.3×10^{-3}	32×10^{-3}
Half-life ($t_{0.5}$): h	26.87	3.54	0.37
Decimal reduction time (*D*-value): h	89.25	11.76	1.21
Enthalpy (ΔH°): KJ/mol	133.3	133.2	133.2
Free energy (ΔG°): KJ/mol	100.14	95.67	92.28
Entropy (ΔS°): J/mol/K	0.10	0.11	0.12

Table 11, a slight change in the values of ΔG° was associated with an increase in temperature.

Entropy (ΔS°) is an additional thermodynamic parameter incorporating both ΔH° and ΔG° contributions. Entropy is the energy per degree needed for the move from a native to the denatured state. As shown in Table 11, the positive values of ΔS° suggested that the thermal denaturation of the enzyme led to an increase in the disorder or randomness of the system (Mohapatra, 2017).

3.4.4.2. Stability in presence of metal ions and additives. Caseinase activity increased in presence of Ca^{2+} and Mg^{2+} ions by 2.24 and 2.16-fold, respectively (Fig. 6C). On the other hand, the activity was not much affected by Dimethyl sulfoxide (DMSO), Ni^{2+}, Cd^{2+} and Na^{2+}. Caseinase activity was decreased by 61.03% in presence of ethylenediaminetetraacetic acid (EDTA) as a metalloprotease inhibitor. Whereas in the presence of Fe^{2+}, phenylmethylsulfonyl fluoride (PMSF) and sodium dodecyl sulfate (SDS) it was completely inhibited. Meshram et al. (2017) reported that protease lost its all activity in the presence of EDTA and ferrous ion.

3.5. Applications of B. megaterium 314 caseinase

3.5.1. Evaluation of its proteolytic activity

3.5.1.1. Decomposition of ES and other protein substances. Most native proteins do not provide desirable functional parameters for food industries. Protein is usually modified by treatment with chemicals or enzymes to improve its functional properties, especially solubility (Onsaard, 2012). Enzymatic treatment for modification is favorite because the process condition is mild (atmospheric pressure and moderate temperature). Modification of protein by proteolytic enzyme is an effective way to enhance its application fields. As shown in Fig. 7 the highest protein content 17.6 and 19.6 mg/g sesame was obtained from Sesame hydrolysate after 24 and 48 h, respectively. This result might be due to the hydrolysis of Sesame protein under alkaline pH with alkaline protease. Singharaj and Onsaard (2015) reported that Sesame protein can be obtained by alkaline extraction with NaOH. Sesame seed (*Sesamum indicm* L.) which has been cultivated in Africa is a good source of proteins ranged from 20 to 25% (Singharaj and Onsaard, 2015). In addition, produced caseinase was able to hydrolyze both OP and LP giving protein 19.2 and 16.3 mg/g substance, respectively after 48 h.

3.5.1.2. Central composite design for optimization of proteolysis of sesame by B.megaterium alkaline protease. The interaction between the three variables (caseinase units, sesame weight and incubation time) was clearly illustrated by counter plot Fig. 8 (a, b, c) (non-linear). As Shown in Table 8 the highest proteolysis 65.98 mg/ml was achieved by adding 750 unit of *B.megaterium* caseinase to 2.5 g Sesame for 96 h in trial number 10 causing 16.85-fold increase if compared with the unoptimized conditions (3.92 mg/ml). The proteolysis (mg/ml) can by calculated from the following equation:

Protein content (mg/ml) = +33.39 + 1.26 * protease units +7.33 * Seasame weight +19.85 * incubation time −1.71* protease units * Seasame weight +0.11* protease units * incubation time +2.02 * Seasame weight * incubation time

The effectiveness of the design was indicated by analysis of variance as shown in Table 9. The success of the design was confirmed by the value of R^2 0.9914 indicated that 99.14% of the variability in the response could be explained by the model. The Predicted R^2 value of 0.9857 was in reasonable agreement with the adjusted R^2 value of 0.9874. The model F-value of 249.20 indicateded the model was significant.

3.5.2. Biological activities

In this part of study, there was an important assay to evaluate the anticoagulation and fibrinolytic activities of the different samples. The results showed promising biological activities (anticoagulation and fibrinolytic) of enzyme preparations.

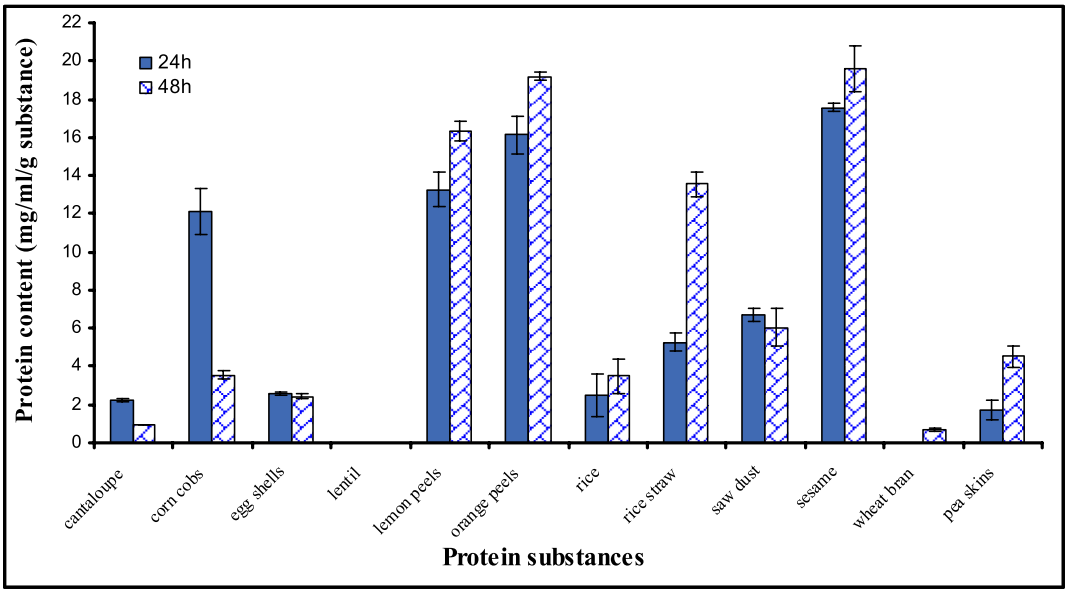

Fig. 7. Proteolysis of ES and other protein substances by *B.megaterium* 314 caseinase.

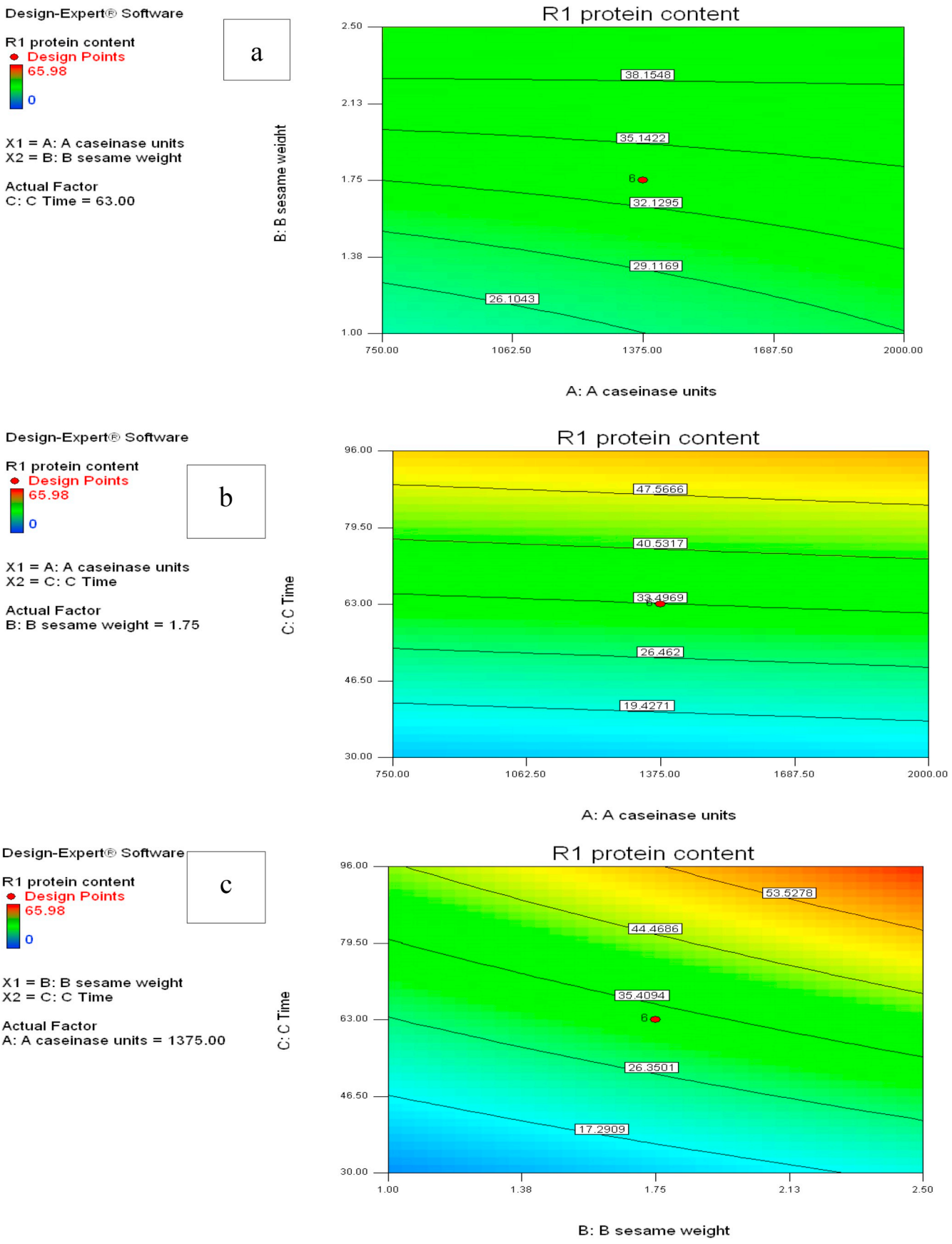

Fig. 8. Contour plot showing combined effect of (a) caseinase units and sesame weight (b) caseinase units and incubation time (c) sesame weight and incubation time on proteolysis.

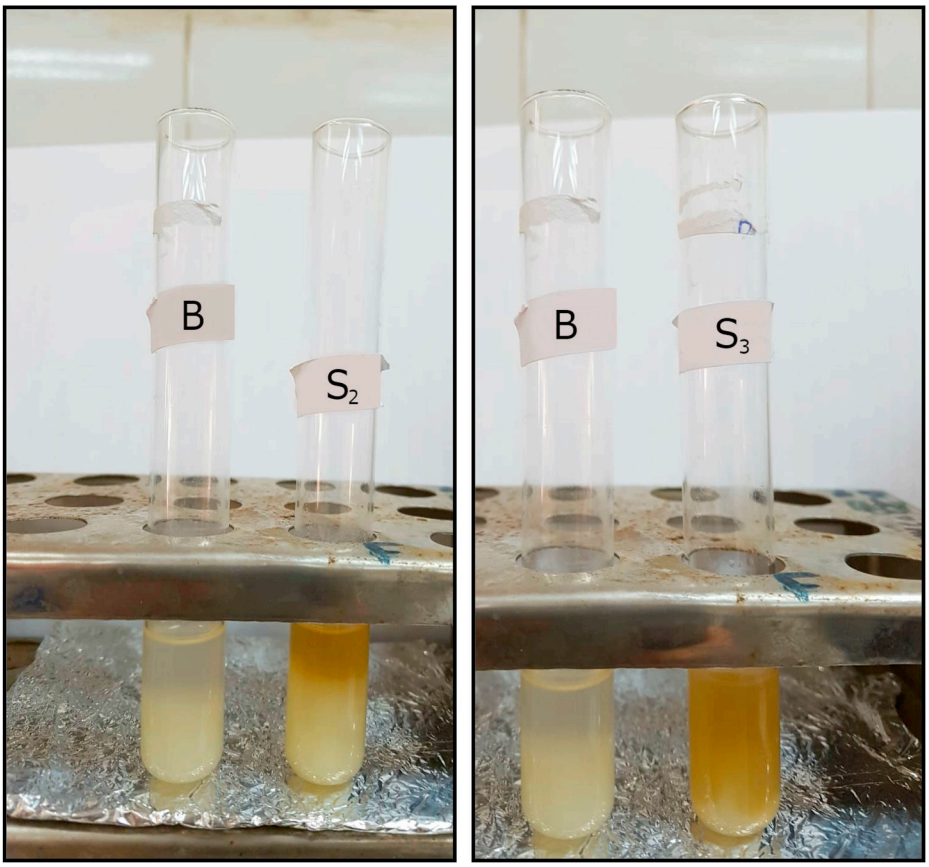

Fig. 9. *In vitro* testing of fibrinolytic activity where: B is the control, S_2 is the media and S_3 is the crude caseinase enzyme.

3.5.2.1. In vitro testing of anticoagulation activity. In this part of study, there was an important assay to evaluate the anticoagulation activity of different samples. The results indicated that both media and crude enzyme have no anticoagulation activities. The positive sample (partial purified enzyme) showed clotting time 24 h comparable to that of standard preparation of heparin sodium. In addition, using diluted partial purified enzyme preparations (10, 100, and 1000 times) had no anticoagulation activities. Inhibition of blood coagulation can be used as an important means to prevent formation of thrombosis Zong et al. (2017). The activation of the anticoagulation system is an important mechanism for antithrombosis.

3.5.2.2. In vitro testing of fibrinolytic activity. The results indicated that most of the samples have no fibrinolytic activities. As shown in Fig. 9 crude enzyme showed high fibrinolytic activity (+8) which is 2-times higher than that obtained by Hemoclar preparation as standard (+4). Thus, it is clearly that the enzyme possesses superior thrombolytic activity under *in vitro* conditions. To our knowledge, this is the first report investigate the anticoagulation and thrombolytic activities of caseinase enzyme which was produced by bacterial strain grew on ES and MS.

4. Conclusions

To the best of our knowledge, this was the first report on the production of casinase enzyme by *B. megaterium* 314 with biological activities utilizing molokihya stalk and egg shell wastes. This study provided an overview of the benefit of animal by-products (ES) with a high proportion of Ca^{+2} and plant waste (MS) with a high proportion of nutrients. Optimized ES pretreatment with HCl enhanced enzyme production by 3.28-fold by utilization of pretreated ES with its extract. In addition, the optimized production medium using PB followed by CC design enhanced enzyme production by 1.78 and 2.47-fold, respectively compared to initial medium. Characterization of caseinase referred to high thermal resistance Enzyme had low *Ea* indicated that the enzyme had massive catalytic efficiency. Calculated thermodynamic parameters indicated the applicability of produced caseinase for industrial processes. Sesame hydrolysate protein by protease enzyme gave the highest protein content 17.6 and 19.6 mg/g after 24 and 48 h, respectively. Further, caseinase enzyme produced by *B. megaterium* 314 had biological activities (anticoagulation and thrombolytic).

Conflicts of interest

The authors declare that they have no conflict of interest.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bcab.2019.101356.

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