



Production, characterization and optimization of fibrinolytic protease from *Bacillus pseudomycooides* strain MA02 isolated from poultry slaughter house soils

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ABSTRACT

Bacterial fibrinolytic enzyme possessed significant application in treatment and prevention of cardiovascular diseases, thus isolation of potential strains producing more fibrinolytic enzyme is of prime importance. In this present study, microorganisms were isolated from soil samples and sediments around poultry slaughter houses and were screened for protease activity. Positive protease producers were further screened for fibrinolytic activity and its efficacy were assessed by fibrinolytic enzyme assay. One strain showed maximum activity of 79.83 FU/mL and the strain was identified as *Bacillus pseudomycooides* strain MA02. The molecular weight of the protease was estimated to be 35 kDa. Response Surface Methodology (RSM) and Central Composite Rotary Design (CCRD) were used to optimize skimmed milk agar to study the effects of the components in the production of fibrinolytic enzyme by *B. pseudomycooides*. The highest enzyme activity of 284 FU/mL was obtained at 7.5 g/L of peptone, 45 g/L of sodium chloride and 5 g/L of skimmed milk, which was 3 fold higher than the un-optimized medium.

1. Introduction

About 17.7 million people/year are dying due to Cardiovascular Diseases (CVDs), which is the major cause of death globally (World Health Organization, 2017). Enzyme therapies are being practiced widely to treat CVDs. Enzymes with anticoagulant, fibrinolytic, anti-inflammatory, mucolytic, antimicrobial, thrombolytic activity were used for therapy. Sumi et al. (1980) reported urokinase obtained from human urine for treatment of acute thrombosis. Nevertheless, these agents are very costly and have some side effects like bleeding complication, low fibrin specificity, and short half-lives (Mackman, 2008). Microorganisms are vital assets for thrombolytic agents. Clinical research has revealed that the greatest approach to therapeutic thrombolysis (clots termination) is an intravenous injection of an enzyme which is competent to convert plasminogen to plasmin that is able to break up the clot (Cichoke, 1990). Fibrinolytic enzymes can be used for digesting fibrin and prevents coagulation process, where fibrin

generated from fibrinogen involves in blood clot (Lioudaki, 2010 and Voet and Voet, 1990). A wide variety of fibrinolytic enzymes such as tissue plasminogen activator (t-PA), urokinase plasminogen activator (u-PA), and bacterial PA (streptokinase) have been widely studied and used as thrombolytic agents (Mukhametova et al., 2001). Enzymes produced by marine microorganisms can provide numerous advantages over traditional enzymes due to the activities at wide pH and temperature ranges (Kim et al., 2009) and can catalyze various biochemical reactions (Mahajan et al., 2012). Many marine microorganisms were subjected for the production and characterization of fibrinolytic enzymes (Huang et al., 2013). Economical and renewable fibrinolytic enzyme producing organisms can be isolated from soil (Ju et al., 2012). However, there are very little studies to inspect these claims (Bang et al., 2014). Discovery of new sources of fibrinolytic enzyme producing bacteria offers an alternative method to synthesis the enzyme which can be used in various field like treating CVDs, myocardial infarction (MI) and others. The overall cost of enzyme production is one of the major

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Table 1
Sources and characteristic of isolates.

Serial No.	Samples code	Sources	Strain number	Number of isolates	Positive protease activity	Fibrinolytic activity
1.	PWSS	Poultry waste soil sample	PWSS 1(1), PWSS 1(2), PWSS 2, PWSS 3(2). PWSS 4(1), PWSS 5(1), PWSS 5(1/2), PWSS 5(3), PWSS 7(1), PWSS 7(2), PWSS 8(1), PWSS 8(2), PWSS 9(1), PWSS 9(2/1), PWSS 9(2/2) 1, PWSS 9(2/2) 2, PWSS 9(2/2) 1(2), PWSS 9(2/2), 1(3)	18	13	5
2.	PW	Poultry waste sediment	PW 1, PW 2, PW 3, PW 4(1), PW 4(2), PW 5, PW 6, PW 7(1/1), PW 7(2/1), PW 7(2/3)	10	5	3
3.	SZNG	Soil from animal housing	SZNG 1, SZNG 2, SZNG 3, SZNG 4, SZNG 5, SZNG 6(1), SZNG 6(2/1), SZNG 6(2/2)	8	5	3
Total				36	23	11

Table 2
The variables and their level for central composite experimental design.

Name	Symbol	Code level				
		- α	-1	0	+1	+ α
Peptone	A	0.80	2.5	5	7.5	9.20
NaCl	B	18.18	25	35	45	51.82
Skim Milk	C	1.59	5	10	15	18.41

challenges against the cost-effective industrial application of enzymes. It is essential to perk up the enzyme production activity in order to increase the product yield without raising the production cost. Enhancement of enzyme production helps to offer a good quality of enzyme productivity but also aids in meeting the emergent demands of enzymes in the industry. Although many fibrinolytic enzymes have been purified and characterized; only very few reports are available concerning culture medium optimization by statistical approach. Response surface methodology is the powerful tool for optimized production of enzymes (Govarthanam et al., 2014).

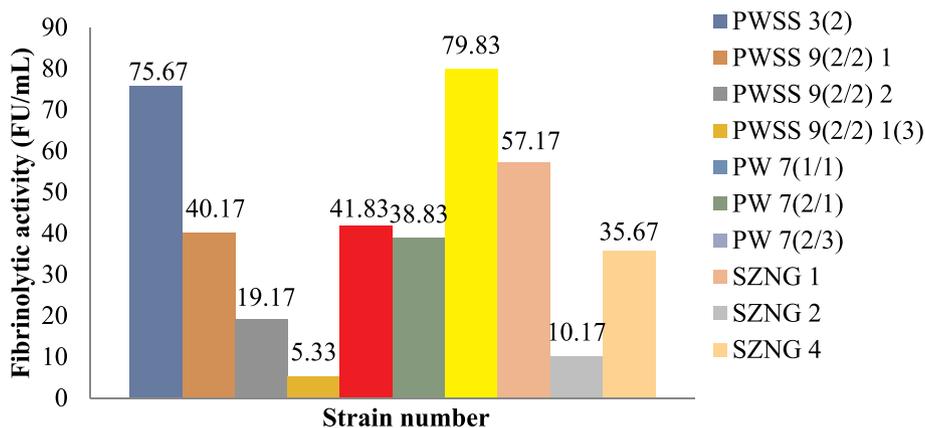


Fig. 1. Fibrinolytic activity of various strains.

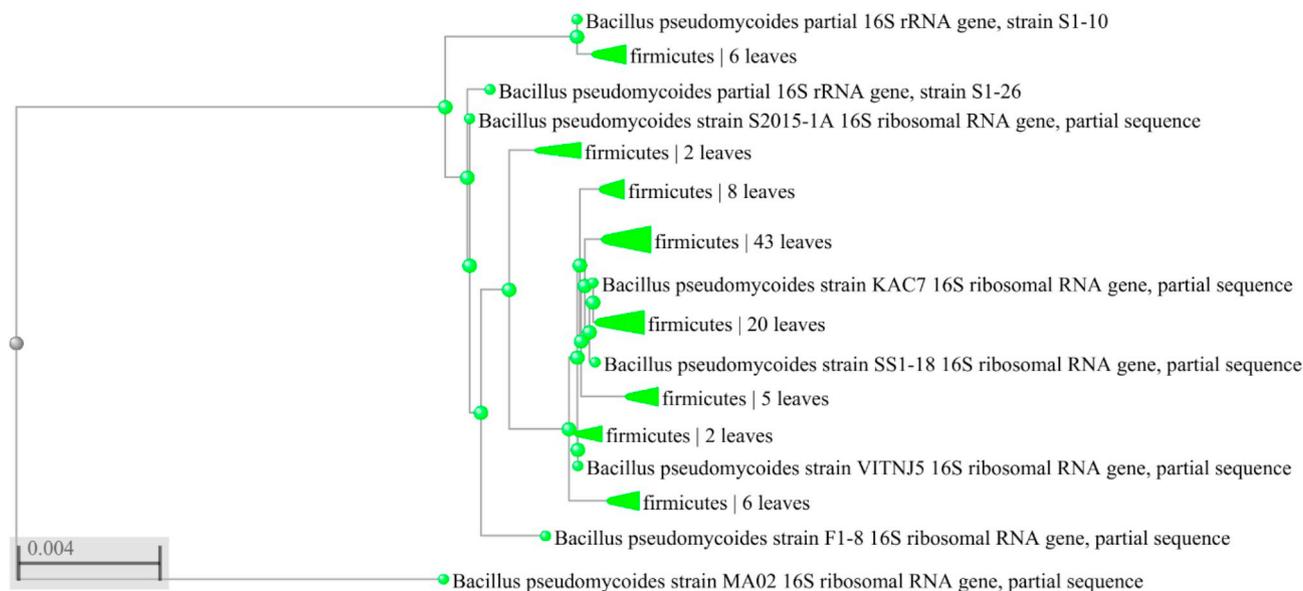


Fig. 2. Phylogenetic tree of *Bacillus pseudomycooides* strain MA02.

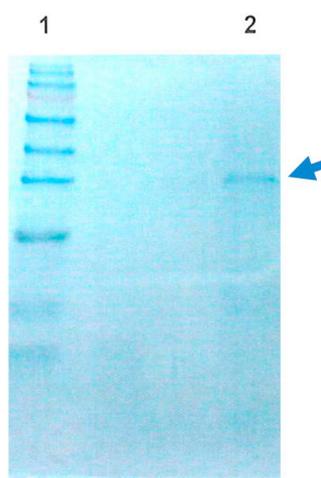


Fig. 3. SDS PAGE image of partially purified enzyme extract. Lane 1: protein marker (10–180 KDa), Lane 2: Partially purified enzyme (10 μ l). arrow denotes the 35 KDa protein.

Table 3
Experimental design and results of the central composite design.

Run	Concentration (g/L)			Fibrinolytic protease activity (FU/mL)
	Peptone	Sodium Chloride	Skimmed milk	
1	5.0	35.0	10.0	79.0
2	5.0	18.2	10.0	50.0
3	7.5	25.0	15.0	75.0
4	5.0	35.0	10.0	79.0
5	5.0	35.0	1.6	42.0
6	0.8	35.0	10.0	70.0
7	2.5	25.0	15.0	55.0
8	7.5	25.0	5.0	62.0
9	5.0	35.0	10.0	79.0
10	5.0	35.0	18.4	40.0
11	5.0	35.0	10.0	79.0
12	2.5	45.0	5.0	82.0
13	5.0	35.0	10.0	79.0
14	5.0	51.8	10.0	100.0
15	5.0	35.0	10.0	79.0
16	2.5	25.0	5.0	32.0
17	7.5	45.0	15.0	125.0
18	9.2	35.0	10.0	87.8
19	2.5	45.0	15.0	43.0
20	7.5	45.0	5.0	284.0

Table 4
Results of the regression analysis of the central composite rotatory design.

Source	Mean Square	F-value	p-value	
Model	4522.017	3.743	0.026	significant
A-Peptide	9701.367	8.030	0.018	
B-NaCl	11372.066	9.413	0.012	
C-Skim Milk	2002.300	1.657	0.227	
AB	6844.500	5.665	0.039	
AC	2112.500	1.749	0.216	
BC	6844.500	5.665	0.039	
AA ²	596.562	0.494	0.498	
BA ²	367.405	0.304	0.593	
CA ²	700.442	0.580	0.464	
Lack of Fit	2416.276			Not significant

In this study, several poultry soil samples were screened for fibrinolytic enzyme producing microorganisms, highest enzyme producing organism was identified and optimization of enzyme production was done by Response Surface Methodology (RSM).

2. Materials and method

2.1. Sample collection

Three samples around poultry slaughter house area located at Lingkar Dagang Mas, Taman Mas, Puchong 47100, Selangor, Malaysia were collected. Samples were collected using sterile spatula in a sterile container and labelled accordingly (Table 1). Containers were brought to the laboratory for further studies.

2.2. Isolation and screening of bacterial strains for protease activity and fibrinolytic activity

One g of soil sample was serially diluted and spread plate was performed on Starch Casein Agar. All plates were incubated overnight at 37 °C. Colonies observed on starch casein agar were streaked on starch casein agar to obtain pure culture (Gopinath and Lingappa, 2016). Obtained culture were inoculated to sterile nutrient agar slant and stored in refrigerator at 4 °C. Isolated pure bacterial colony was streaked on skimmed milk agar plate. Colonies with zone of clearance were further selected for screening fibrinolytic activity (Vijayaraghavan and Vincent, 2014). Positive protease organisms were streaked on fibrin plate containing 2% agarose (w/v), 0.08% human fibrinogen (w/v), and thrombin (100 NIHU/mL) (Ohta et al., 1972). The fibrin plate was allowed to stand for 1 h at room temperature to form a fibrin clot layer. Holes were made on the fibrin plate using a gel puncture. 10 μ L of the crude enzymes from the bacterial isolates was dropped into the wells and incubated at 37 °C for 24 h. The diameter of the clear zone was measured (Astrup, and Mullertz, 1952). Samples with positive fibrinolytic activity were confirmed and were preserved in nutrient agar with 70% glycerol (Vijayaraghavan et al., 2017).

2.3. Fibrinolytic protease assay

Fibrinolytic protease activity was evaluated according to the fibrin degradation assay with slight modification (Wang et al., 2009). Culture was centrifuged at 10000 rpm, pellet was subjected for drying. Culture supernatant (0.1 mL) was mixed with 2.5 mL of 0.1 M Tris-HCl buffer (pH 7.8) containing 0.01 M calcium chloride. To this, 2.5 mL of fibrin suspension (1.2%, w/v) was added and incubated at 37 °C for 15 min. The reaction was stopped by adding 5.0 mL of 0.11 M trichloroacetic acid containing 0.22 M sodium acetate and 0.33 M acetic acid. The absorbance was measured at 275 nm against sample blank. Fibrinolytic protease unit was determined by the absorbance increase at 275 nm which is equivalent to 1 μ g of tyrosine/min at certain temperature (Vijayaraghavan and Vincent, 2014). Enzyme activity was calculated based on given formula (Wang et al., 2009).

$$\text{Enzyme activity (U/mL)} = [(OD_s - OD_c) / (0.01 \times 60 \times 0.1)] \times (V / W)$$

Where OD_s was optical density value of sample, OD_c is optical density value of control, V is total volume and W is dry weight of organisms.

2.4. Identification of bacterial strain

The selected bacteria were identified based on cell morphology, grams stain, biochemical tests (Mcclung et al., 1987) and 16S rRNA sequencing.

2.5. Characterization of fibrinolytic protease

Sample was centrifuged at 10,000 rpm for 10 min, pH of collected supernatant was adjusted to 7.0. Solid ammonium sulphate $(\text{NH}_4)_2\text{SO}_4$ was dissolved to obtain 30% saturation and kept overnight. Precipitate was collected by centrifugation at 10,000 rpm for 10 min at 4 °C. Supernatant obtained was adjusted to pH 8.6 and kept overnight at 4 °C

Design-Expert® Software
 Trial Version

Factor Coding: Actual

Fibrinolytic activity (FU/mL)

● Design points above predicted value

32 284

X1 = A: Peptone

X2 = B: NaCl

Actual Factor

C: Skim Milk = 5

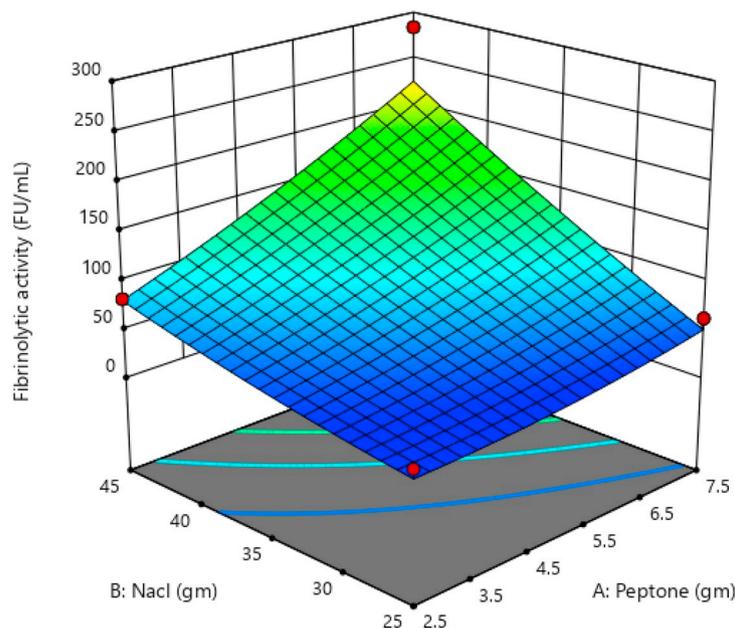


Fig. 4. Response surface plot for fibrinolytic protease activity by *B. pseudomycolides* when skimmed milk is 5 g/L.

Design-Expert® Software
 Trial Version

Factor Coding: Actual

Fibrinolytic activity (FU/mL)

● Design points above predicted value

32 284

X1 = A: Peptone

X2 = B: NaCl

Actual Factor

C: Skim Milk = 10

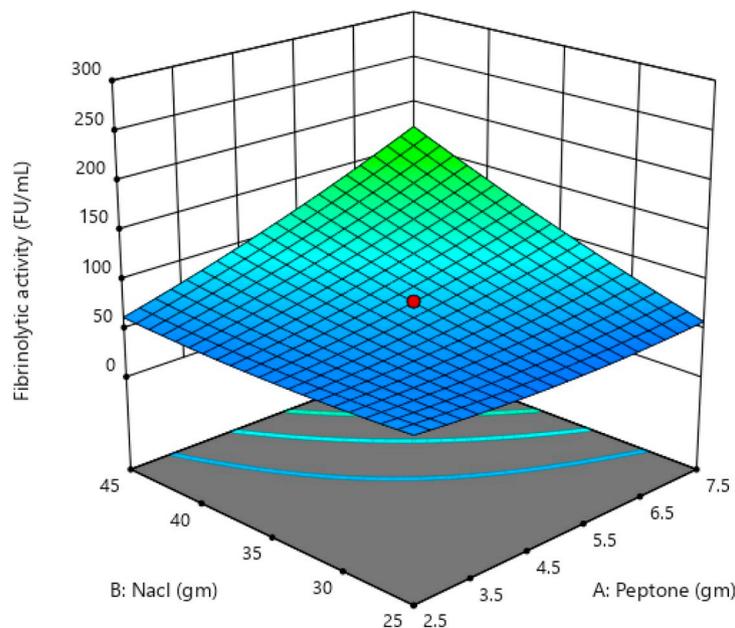


Fig. 5. Response surface plot for fibrinolytic protease activity by *B. pseudomycolides* when skimmed milk is 10 g/L.

(Hao et al., 2002). Then, it was dissolved in PBS buffer and 10 µL of sample was loaded onto fibrin plate assay for determination of the fibrinolytic activity (Bin et al., 2013). Aliquot obtained from ammonium sulphate precipitation technique was subjected for dialysis (Sasirekha et al., 2012) using Maxi Pur-A-Lyzer (50 kDa MVCO) (Sigma-Aldrich). 12% polyacrylamide gel with 1% SDS was used for protein separation

(He, 2011) Gel was stained with Coomassie Brilliant Blue R-250 after electrophoresis.

2.6. Optimization of medium for fibrinolytic protease production

Media components for the production of protease using the isolate

Design-Expert® Software
 Trial Version
 Factor Coding: Actual

Fibrinolytic activity (FU/mL)
 ● Design points above predicted value
 ○ Design points below predicted value
 32  284

X1 = A: Peptone
 X2 = B: Nacl

Actual Factor
 C: Skim Milk = 15

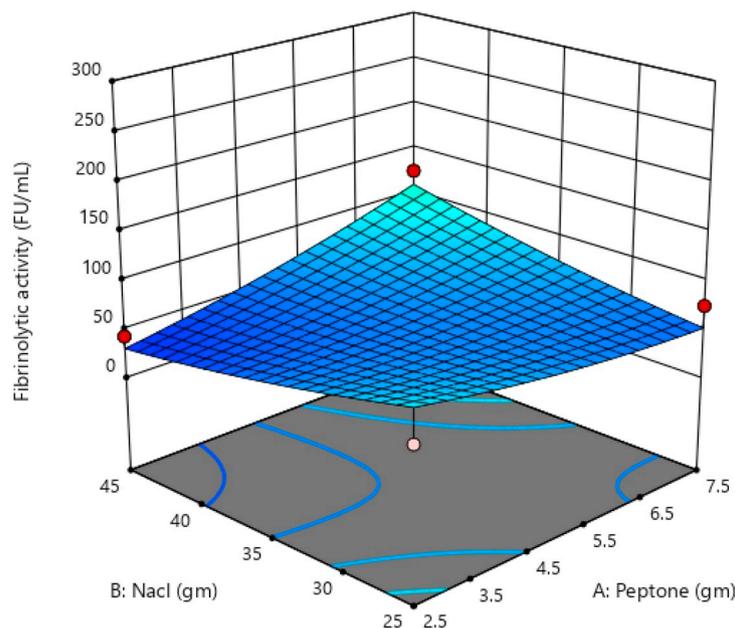


Fig. 6. Response surface plot for fibrinolytic protease activity by *B. pseudomycooides* when skimmed milk is 15 g/L.

Design-Expert® Software
 Trial Version
 Factor Coding: Actual

Fibrinolytic activity (FU/mL)

Actual Factors
 A: Peptone = 5
 B: Nacl = 35
 C: Skim Milk = 10

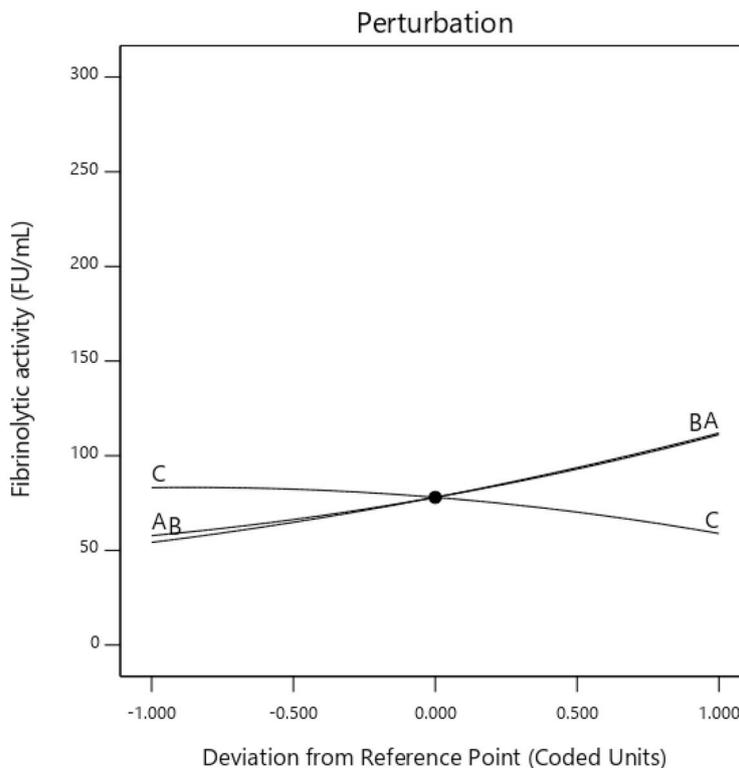


Fig. 7. Perturbation graph showing the effects of each of the independent variables on fibrinolytic protease production while keeping other variables at their respective midpoint level. (A) Peptone, (B) Sodium chloride and (C) Skimmed milk.

were optimized by Factorial Central Composite Rotatory Design (FCCRD). In which the media components concentration was taken as variable, other variables like pH, temperature and duration for culture are kept constant. Factorial Central Composite rotatory design (CCRD)

for 3 factors with 6 replicates at central value or start points was used as experimental design in this study. The variables were peptone, sodium chloride and skimmed milk, each factor with 5 code level: α , -1, 0, +1 and $+\alpha$ as shown in Table 2. The values of variables for CCRD were

selected based on the initial culture medium composition which acts as the central point in this experimental design. This CCRD consist of 20 experimental trials in total with 6 trials as central point and 14 trials as factorial designs. The response value, Y, in each trial was average of duplicates in each trial. The results of the experimental design were analysed and interpreted using Design Expert Version 7.1.5 (stat-Ease Inc., Minneapolis USA) statistical software.

3. Result and discussion

Microorganisms are essential assets for thrombolytic agents because bacterial fibrinolytic proteases are considered as the harmless thrombolytic agent. Moreover, administration of these agents upon oral administration could boost fibrinolytic activity in human plasma (Sumi et al., 1990). Mander et al. (2011) also mentioned that enzymes produced from soil bacteria are capable to offer plentiful benefits over conventional enzymes due to the extensive range of environments from where they are recovered.

In the present study soil samples from nearby slaughter house area were chosen since bacteria which are present or isolated from this area have the higher possibility to produce fibrinolytic protease which can degrade fibrin. Also, bacterial strain isolated from this source can easily reproduce. As eloquently stated by Haas et al. (2005), concentration of microorganisms in poultry slaughter house appears higher than the composting plants.

In this study, total of 36 bacterial strains were isolated from three different soil samples. 23 out of 36 isolates showed positive protease activity (Table 1). Fibrinolytic proteases is a subclass of proteases and competent to demean fibrin substrate (Fujita et al., 1993). Monod et al. (1991) stated that it has the capability to breakdown proteins like fibrin, fibrinogen, elastin, collagen or laminin. Streptokinase and Staphylokinase are reported to be used in thrombolytic therapy (Collen and Lijnen, 1994). From 23 strains, 11 strains were found to be positive for fibrinolytic protease production and its activity ranged from 5.33 to 79.83 FU/mL (Table 1 and Fig. 1). In the midst of 11 isolates, PW 7(2/3) strain isolated from soil sediment showed the greatest zone of lysis with greatest fibrinolytic activity of 79.83 FU/mL. Thus PW 7 (2/3) strain was further subjected for identification, and it was identified on its biochemical, cultural, staining properties and 16S rRNA sequencing as *Bacillus pseudomycoloides* strain MA02. Sequence was submitted in GENBANK (Accession number: MK590245) (Fig. 2).

Partially purified fibrinolytic protease showed enzyme activity of 252.33 FU/mL. and the enzyme demonstrated a clear band matching to molecular weight of 35 kDa (Fig. 3). Previous study reported by Gu et al. (2009) stated that a single protein fraction was acquired from *Bacillus pseudomycoloides* B-60 where a single band was found in the SDS-PAGE with a relative molecular weight of 34 kDa. The molecular weight of protein from *Bacillus pseudomycoloides* B-60 was almost equivalent with fibrinolytic proteases from *Bacillus pseudomycoloides* PW 7(2/3).

The Experimental design and results of the central composite design were as shown in Table 3. Where P value is less than 0.05, it indicated that the model term is significant in the study. In this study, linear term A and B, quadratic term AB and BC are shown to be statistically significant in Table 4. The statistical significance of the model equation was evaluated by F-test for analysis of variance (ANOVA), as shown in Table 4, which shows a 95% ($p < 0.05$) confidence level. The model F-value of 3.74 for fibrinolytic protease activity implies that this model is significant. There is a 2.58% chance that an F-value this large could occur due to noise. The value of $p > F$ less than 0.05 also indicates that the model is also significant. The coefficient of determination (R^2) was calculated with the value of 0.7711 indicates that there are 77% of the variability could be explained in this model. According to ANOVA, the lack of fit is insignificant, indicating that the second-order model with interaction is very adequate in approximating the response surface of the experimental design. Adeq. Precision" measures the signal to noise ration. The desired ratio for this is 4. In this study, the ratio is 8.220

indicated an adequate signal. Therefore, this model is significant in this aspect. A three dimensional response surfaces was plotted using model equation, to study the interaction between variables and determine the optimum concentration for each factor in the medium for maximum production of fibrinolytic protease in *B. pseudomycoloides*. In this study, it is seen that various concentration of peptone and sodium chloride had shown an effect on the production of fibrinolytic protease in *B. pseudomycoloides* shown in Fig. 4, Fig. 5, and Fig. 6 which demonstrate similar pattern with different concentration of skimmed milk: 5 g/L, 10 g/L and 15 g/L. it is noticed that as the concentration of both the concentration of peptone (A) and sodium chloride (B), there is an increase in fibrinolytic protease activity. Thus, indicating more fibrinolytic protease was produced by *B. pseudomycoloides* strain MA02. When skimmed milk (C) concentration is 15 g/L, peptone and sodium chloride present either in low (2.5 g/L and 25 g/L respectively) or high (7.5 g/L and 45 g/L respectively) concentration had an increase in fibrinolytic protease activity. When skimmed milk concentration is 10 g/L, fibrinolytic protease activity increases as the concentration of both peptone and sodium chloride increases. Fibrinolytic protease activity remains almost constant when only either peptone or sodium chloride concentration increase. When skimmed milk concentration is 5 g/L there is dramatic increase in fibrinolytic protease activity when both peptone and sodium chloride concentration increase. Based on the response surface and perturbation graph (Fig. 7), it is noticed that the major factor that cause increase in fibrinolytic protease production is peptone and sodium chloride. Fig. 7 shows, that skimmed milk did not have any significant in fibrinolytic protease production. Peptone and sodium chloride are the major factor that contributes to fibrinolytic protease production. The maximum fibrinolytic protease activity is 284 FU/mL, with the medium component concentration at: peptone (7.5 g/L), sodium chloride (45 g/L) and skimmed milk (5 g/L) which was about 3 fold of the original un-optimized medium, 79 FU/mL. Using the RSM method, reported that increase of protease by 1.5 and 1086 fold in *Bacillus aquimaris* VITP4 (Chittoor et al., 2016) and *Penicillium bilaiae* (Mefteh et al., 2019) respectively.

4. Conclusion

In this study, poultry soil samples were used for isolation of fibrinolytic enzymes. Amongst 36 microorganisms, the highest enzyme activity possessing strain was chosen for optimization study and that strain was identified to be *Bacillus pseudomycoloides* strain MA02. The molecular weight of enzyme was found to be of 35kDa. Media was optimized using RSM and the enzyme production was found to be increasing three fold at 7.5 g/L of peptone, 45 g/L of sodium chloride and 5 g/L of skimmed milk. Therefore, the fibrinolytic protease production by *Bacillus pseudomycoloides* strain MA02 would serve as a good source for further therapeutical approaches.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.bcab.2019.101371>.

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