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Research Article

Enhanced Production of Fibrinolytic Enzyme from *Bacillus amyloliquefaciens* CGMCC 7380 Using Broad Bean (*Vicia faba* L.) as Substrate

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Abstract: Fibrinolytic enzyme is a potent protease with the potential for thrombolytic therapy. In this research, liquid fermentation production fibrinolytic enzyme by a newly isolated *Bacillus amyloliquefaciens* CGMCC 7380 using broad bean (*Vicia faba* L.) as substrate was investigated for the first time. With the *B. amyloliquefaciens* CGMCC 7380, the design of the optimum medium composition for high-level production of broad bean-fibrinolytic enzyme was attempted by using Plackett-Burman design and Central Composite Design. Maximum protease activity (4900 U/mL) was obtained when the strain was grown in medium containing (g/L): broad bean power 38.0, maltose 32.0, $CaCl_2$ 0.2, K_2HPO_4 2.0, KH_2PO_4 2.0, $MgCl_2$ 0.5. Furthermore, *in vitro* studies revealed that the enzyme could catalyze fibrin clot lysis effectively. And in carrageenan induced mouse tail thrombosis model, tail-thrombus of the enzyme treated group was significantly shorter than the control group indicated that the broad bean-fibrinolytic protease could be a useful thrombolytic agent. Therefore, this study provided a novel method for the preparation of functional food of broad bean and potential thrombolytic drugs.

Keywords: *Bacillus amyloliquefaciens* CGMCC 7380, broad bean (*Vicia faba* L.), fibrinolytic enzyme, thrombolytic activity

INTRODUCTION

Stressful era of modernization has lead to high rates of cardiovascular diseases (CVDs) globally (Mahajan *et al*., 2010). Intravascular thrombosis due to accumulation of fibrin in blood vessel is one of the major causes of CVDs (Peng *et al*., 2005). Thrombolytic therapies via injecting or orally administrating fibrinolytic enzymes have been widely used and these products in current clinical usage are plasminogen activators (PA) which require plasminogen to degrade fibrin (Wang *et al*., 2008; Singh *et al*., 2014). However, these agents exhibit many shortcomings such as their expensive prices, low specificity to fibrin, cause hemorrhagic complication and has a short half life (Singh *et al*., 2014). Therefore, search for the safer, cost effective and more efficient fibrinolytic proteases is of paramount necessity.

Currently, in order to produce more cheaper and effective fibrinolytic enzymes, various crude substrates have been reported to serve as excellent carbon or nitrogen source for fibrinolytic protease production by *Bacillus* spp. (Ku *et al*., 2009; Wei *et al*., 2011; Chang *et al*., 2012). However, no study has focused on producing fibrinolytic enzyme with *Bacillus* spp. using broad bean as substrate. Broad bean (*Vicia faba* L.) originated in the Mediterranean, is now grown in many parts of the world (Sofi *et al*., 2013). Due to their high nutritive and high protein content (Lisiewska *et al*., 2007), broad bean is honored as the new origin of plant protein (Wang *et al*., 2010). However, broad bean protein are usually used in the snack food industry (Giménez *et al*., 2013). Furthermore, chemical analysis showed that broad bean are rich in organic and inorganic compounds, suggesting it as a good candidate for culture medium.

In this study, we attempted to use broad bean as a new food grade microbial growth substrate for production of fibrinolytic protease by *Bacillus amyloliquefaciens* CGMCC 7380 and the stepwise optimization was performed including one-at-a-time method, Plackett-Burman design and Central Composite Design. Moreover, the thrombolytic activity of broad bean-fibrinolytic enzyme *in vitro* and *in vivo* were also investigated. From these results indicated that *Bacillus amyloliquefaciens* CGMCC 7380 as a food source microbial alternative for broad bean fermentation for the production of fibrinolytic enzyme not only enhance the activity of fibrinolytic enzyme, but also promote the economic value of the broad bean.

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MATERIALS AND METHODS

Materials: Bovine fibrinogen, bovine thrombin, Folin-Phenol reagent, Carrageenan (Type I, C1013) were obtained from Sigma. Other reagents and chemicals used were of analytical grade and were commercially available.

To obtain broad bean flour (BBF), raw material was soaked, dehulled and then dried at 50°C. The dried preparation was grined and sieved to obtain a fine powder and then stored in glass bottles at room temperature. The BBF contained 29.26±0.196% protein, 1.90±0.078% fat and 2.73±0.007% ash.

Screening of strain with high production of fibrinolytic enzyme: The collected different kinds of soybean fermented samples were boiled for 15 min in normal saline (85°C) and suspension was spread out onto the skim-milk agar medium containing 50 g/L skim-milk and 20 g/L agar. After incubation at 37°C for 24 h, the colonies surrounded by a clear zone were picked up and inoculated into fermentation medium (soybean meal 45.0 g/L, dextrin 20.0 g/L, $MgSO_4 \cdot 7H_2O$ 0.5 g/L, $CaCl_2$ 0.2 g/L, K_2HPO_4 1.0 g/L, KH_2PO_4 6.0 g/L) and further cultivated in a shaking incubator (200 rpm) at 37°C for 48 h. The fibrinolytic activity was measured by fibrin-plate method and casein degradation method. The highest productive strain was identified by API 50CHB (BioMérieux, France) and 16S rDNA sequence. The phylogenetic tree was also constructed with NCBI database.

Measurement of enzyme activity: Proteolytic activity was assayed according to the method of Murata *et al*. (1963) using casein as reaction substrate. Reaction mixture was prepared by mixing 1 mL enzyme sample with 1 mL of 2% (w/v) casein in 20 mmol/L phosphate buffer (pH 7.2). Following incubation at 40°C for 10 min, 2 mL of ice-cold 0.4 mol/L trichloroacetic acid was added and then immersed in ice water for 20 min. After centrifugation at 10000×g for 10 min, the supernatant was mixed with 1 mL Folin-Phenol reagent (0.33 mol/L) and 5 mL Na_2CO_3 (0.4 mol/L) solution. The mixture was incubated at 40°C for 20 min and the optical density of the produced color was measured at 660 nm using spectrophotometer. To assess the enzyme activity, a standard curve was generated using tyrosine solutions with different concentrations, ranging from 0 to 100 μg/mL. Protease activity was quantified in unit (U), where 1U is defined to be the amount of enzyme required to release 1 μg tyrosine per min under the assay condition.

Qualitative analysis of fibrinolytic activity was conducted using fibrin plate, following the method described by Astrup and Mullertz (1952) with slight modifications. Fibrin plate was prepared by pouring the solution composed of 0.75% (w/v) fibrinogen in 10 mmol/L Tris-HCl buffer (pH 7.2), 1.0% (w/v) agarose and 42 U/mL thrombin into the petri dish. The plates were left for an hour at room temperature to form fibrin clot. Ten microliters of the purified enzyme was carefully loaded onto each circular well (2 mm in diameter) on the plate and then the plate was incubated at 37°C. Fibrinolytic activity was estimated by measuring the diameter of the lytic circle around the well.

Table 1: Variables and their levels for Plackett-Burman design

		Level (g/L)	
Variable	Variable code	Low (-1)	High (+1)
BBF	A	40	60
Maltose	B	15	30
$CaCl_2$	C	0.2	0.4
$MgCl_2$	D	0.5	1.0
K_2HPO_4	E	2	4
KH_2PO_4	F	2	4

Selection of the most suitable carbon and nitrogen sources on fibrinolytic enzyme production: Initial screening of the most suitable carbon and nitrogen sources were performed by the one-at-a-time approach. Four different high protein substrate as nitrogen source at concentration of 40 g/L (defatted soybean, broad bean, chickpea, soy meal) and eight carbon sources (20 g/L) include dextrin, glucose, xylose, maltose, sucrose, glycerin, lactose, starch were investigated. Initial medium consists of (g/L): soybean meal 40.0, dextrin 20.0, $MgCl_2$ 0.5, $CaCl_2$ 0.2, K_2HPO_4 1.0, KH_2PO_4 6.0. The above different nitrogen and carbon sources are used instead of the soybean meal and dextrin, respectively. All experiments were carried out in triplicate and repeated at least twice.

Elucidation of significant components with plackett-burman design: The Plackett-Burman design was used to determine which nutrients had a significant effect on fibrinolytic enzyme production. The variable chosen for the study were broad bean, maltose, $MgCl_2$, $CaCl_2$, K_2HPO_4 and KH_2PO_4. Each variable was examined at high levels (coded "+1") and a low setting (coded "-1"). Table 1 lists the variables and levels in detail. Table 2 shows the design matrix and experimental fibrinolytic protease production levels. Design Expert 7.0 (Stat-Ease, Inc., Minneapolis, MN, USA) was used to analyze the experimental Plackett-Burman design.

Optimization of key ingredients with Central Composite Design (CCD): According to Plackett-Burman test results, we used Central Composite Design (CCD) to determine the optimal concentrations of the significant factors. A two-factor-three-level CCD was employed in this study and all 13 of the designed experiments were conducted to optimize the two independent variables. The variables, levels and

Table 2: Plackett-Burman experimental design matrix with fibrinolytic enzyme production levels

Trial	A	B	C	D	E	F	Fibrinolytic activity (U/mL)
1	-1	-1	-1	-1	-1	-1	3418.95
2	1	1	1	-1	-1	-1	3195.79
3	-1	1	-1	1	1	-1	3962.11
4	-1	-1	1	-1	1	1	4202.11
5	1	1	-1	1	1	1	3187.37
6	-1	-1	-1	1	-1	1	4037.90
7	1	-1	-1	-1	1	-1	917.90
8	-1	1	1	-1	1	1	4703.16
9	-1	1	1	1	-1	-1	4218.95
10	1	-1	1	1	-1	1	741.05
11	1	1	-1	-1	-1	1	2126.32
12	1	-1	1	1	1	-1	850.53

Table 3: Variables and their levels used in the CCD experimental design

		Level (g/L)		
Variable	Variable code	-1	0	1
BBF	A	24	32	40
maltose	B	30	34	38

Table 4: Experimental design and results of CCD experimental

Trail	A	B	Fibrinolytic activity (U/mL)
1	32	34	4454.739
2	32	34	4416.844
3	43.3	34	5621.055
4	32	34	5094.739
5	32	28.3	4517.897
6	20.7	34	943.1583
7	40	38	3743.159
8	32	34	4197.896
9	24	30	1646.316
10	40	30	4395.791
11	24	38	2126.317
12	32	39.7	3802.107
13	32	34	5111.581

correspongding experimental results were shown in Table 3 and 4.

Fibrin clot lytic effect of the broad bean-fibrinolytic enzyme *in vitro*: The mixture of 1 mL 0.25% (w/v) fibrinogen and 50 μL 100 U/mL thrombin was incubated at room temperature for 1 h. 20 μL of crude enzyme was added into this solution and then incubated at 37°C.

Thrombolytic activity *in vivo*: Carrageenan-induced mouse tail thrombosis model was used to examine the thrombolytic effect of broad bean-fibrinolytic enzyme *in vivo*. A total of 12 male ICR mice was randomly divided into 2 groups (n = 6). Group 1 served as control with saline water, group 2 was given broad-bean fibrinolytic enzyme. After mouse was subcutaneously injected with κ-carrageenan, crude fibrinolytic protease or saline water was injected through celiac into mice. The thrombus length were measured at 24 h (Yan *et al*., 2009).

RESULTS AND DISCUSSION

Screening of strain with high production of fibrinolytic enzyme: To obtain high producing fibrinolytic enzyme strain, fibrin-plate method and

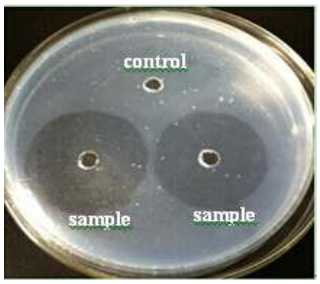


Fig. 1: Identification of fibrinolytic activity produced by strain 553 on a fibrin plate. control: Tris-HCl buffer (20 mmol/L, pH 7.2); sample: fermentation supernatant of strain 553

casein degradation method were used to evaluate the fibrinolytic activity. Among all the proteolytic bacteria screened, the isolate strain 553 exhibited prominent clear zone on the fibrin plate indicating that was secretes fibrinolytic enzyme (Fig. 1). The most productive strain 553 showed fibrinolytic activity of 2590 U/mL, which higher than the fibrinolytic enzyme productive strain-*Bacillus cereus* NS-2 (145.5 U/mL) (Bajaj *et al*., 2013). This strain was further identified based on API experiment and 16S rDNA sequences. The subsequently constructed phylogenetic tree showed that the strain 553 was tightly clustered with *B. amyloliquefaciens* (Fig. 2). From these results, the strain was identified as *B. amyloliquefaciens* and it was deposited at the China General Microbiological Culture Collection Center (CGMCC No. 7380).

Selection of the most suitable nitrogen and carbon sources on fibrinolytic enzyme production: Various high protein substrates have been reported to serve as substrates for fibrinolytic enzyme production, such as defatted soybean-from *Bacillus subtilis* natto (Ku *et al*., 2009), chickpea-from *Bacillus* spp. (Wei *et al*., 2011, 2012), soy meal-from *Bacillus natto* NLSSE (Liu *et al*., 2005). Broad bean is not only rich in protein content (29.26±0.196%), but also contain carbohydrates, minerals and other chemical ingredients. It shows that broad bean could be a suitable ingredient in the medium and production of fibrinolytic protease. Herein, BBF and other substrates were compared as nitrogen sources for production of fibrinolytic enzyme with *B. amyloliquefaciens* CGMCC

Fig. 2: Phylogenetic tree of strain 553 based on 16S rDNA sequence

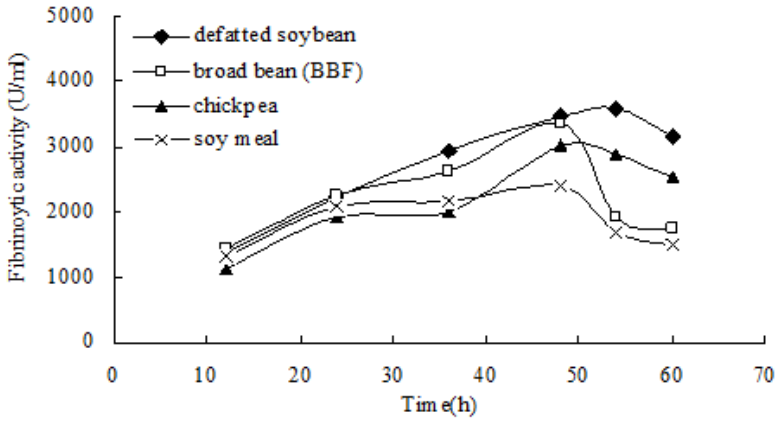


Fig. 3: Effect of different nitrogen sources on protease production by *B. amyloliquefaciens* CGMCC 7380

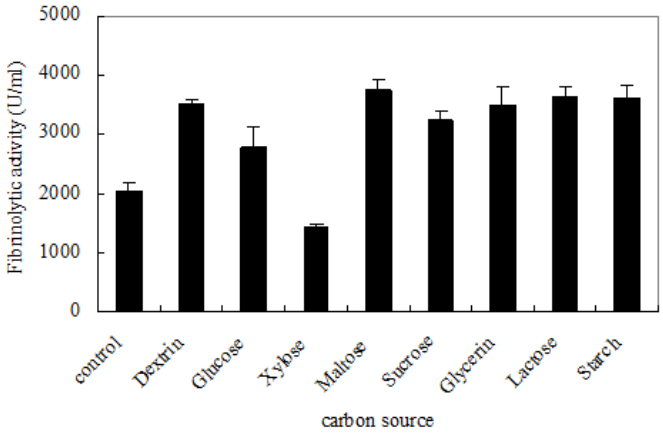


Fig. 4: Effect of different carbon sources on protease production by *B. amyloliquefaciens* CGMCC 7380

Table 5: Identification of significant variables for fibrinolytic enzyme production by *B. amyloliquefaciens* CGMCC 7380 using the Plackett-Burman design

Variable	Effect estimate	Sum of squares	T	Prob>F
A	-2275	217.9	-5.22	0.003
B	1225	217.9	2.81	0.037
C	88	229.3	0.19	0.855
D	-216	229.3	-0.47	0.657
E	-7	217.9	-0.02	0.987
F	427	217.9	0.98	0.372

7380. As shown in Fig. 3, the maximum levels of fibrinolytic activity were carried out with defatted soybean (3612 U/mL) followed by BBF (3364 U/ml), chickpea (3020 U/mL) and soy meal (2394 U/mL). So, the soy meal was not suitable as an inducer for fibrinolytic enzyme production in this study. Furthermore, *B. amyloliquefaciens* CGMCC 7380 produced maximum fibrinolytic enzyme using defatted soybean as nitrogen source appeared on the 54 h, while the optimum protease production was obtained with BBF at 48 h. Therefore, BBF was more suitable as nitrogen source for enhancement of fibrinolytic enzyme production by *B. amyloliquefaciens* CGMCC 7380.

In the present study, different carbon sources at a concentration of 20 g/L were tested in the initial medium containing BBF as nitrogen source. The optimum protease production was obtained with maltose (3747 U/mL) as carbon source (Fig. 4). The results was similar with the observations by Liu *et al*. (2005) and Wang *et al*. (2009) which maltose is the best carbon source for fibrinolytic protease production from *Bacillus* spp. Moreover, the strain could also grown in medium without carbon source, indicated that BBF could also be used as carbon source by the strain.

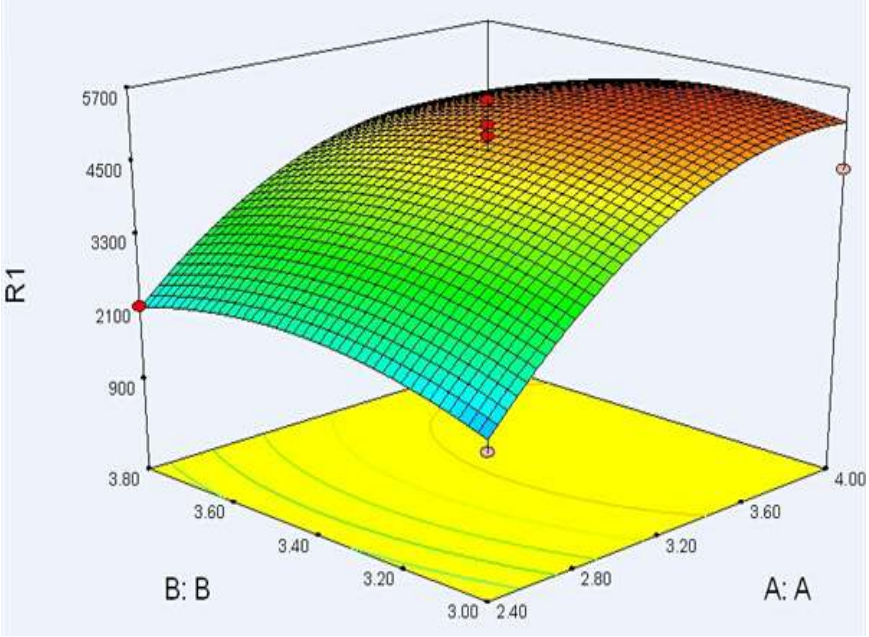
Elucidation of significant components with Plackett-Burman design: Among the various carbon and nitrogen sources tested, maltose and BBF were found to be the most suitable substrates for the production of fibrinolytic activity. Thus, maltose, BBF, $MgCl_2$, $CaCl_2$, K_2HPO_4, KH_2PO_4 were selected for further optimization steps. Firstly, Plackett-Burman design was used for screening the significant components that affect fibrinolytic enzyme production (Table 1 and 2). As shown in Table 5, factors having a confidence greater than 95% (Prob>F<0.05) were considered to have a significant effect on the protease production. From the experimental data analysis, maltose (Prob>F = 0.037<0.05) and BBF (Prob>F = 0.003<0.05) were found to be the most influencing factors in the medium. Moreover, the effect estimate of maltose was positive, which suggested that the increase of its concentration in the medium will resulted in the promotion of protease production, while the effect estimates of broad bean was negative, which meant low level of the broad bean content would benefit fibrinolytic enzyme production process. Therefore, the two nutrient components (BBF and maltose) will be further investigated with central composite design, other variations concentration such as $MgCl_2$, $CaCl_2$, K_2HPO_4 and KH_2PO_4 in medium were selected from one-at-a-time method in previous research.

Optimization of key ingredients with Central Composite Design (CCD): CCD was used to develop a predicted model for optimizing the medium compostion. The experimental conditions and the corresponding values from the experimental design were presented in Table 3 and 4. The ANOVA of the optimization study given in Table 6 showed that the model Prob>F is 0.0033 (Prob>F<0.01), thus proving that the model is significant. And as the test of lack of fit hypothesis was not significant (Prob>F = 0.2320) in model equations, the models were fitted to the fibrinolytic rate data. The regression equation obtained from the ANOVA indicated that the value of R^2 is 0.8872. This value indicated that only 11.28% of the total variation was not explained by the model. In addition, the model has an adequate precision value of 9.058, the adequate precision value is an index of the

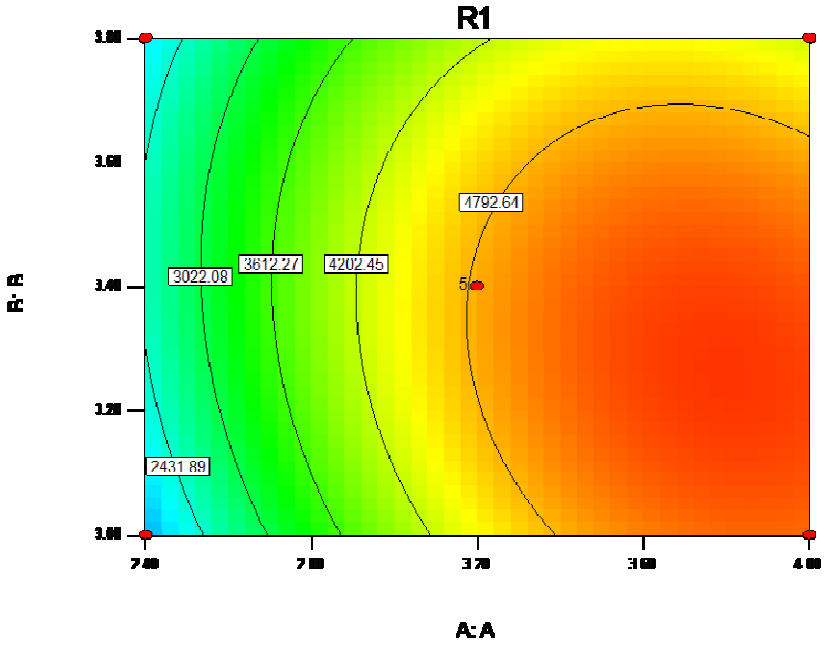
Table 6: ANOVA for the quadratic model

	Degrees of freedom	Sum of squares	Mean squares	F value	Prob>F
Model	5	2.310E+007	4.619E+006	11.01	0.0033
Residual	7	2.937E+006	4.196E+005		
Lack of fit	3	1.825E+006	6.084E+005	2.19	0.2320
Pure error	4	1.112E+006	2.780E+005		
Total	12	2.603E+007			

R^2 = 0.8872; Adj R^2 = 0.8066; Adeq Precision = 9.509



(a)



(b)

Fig. 5: Response surface plot for fibrinolytic enzyme production showing the interactive effects of the BBF (A, %) and maltose (B, %) concentrations

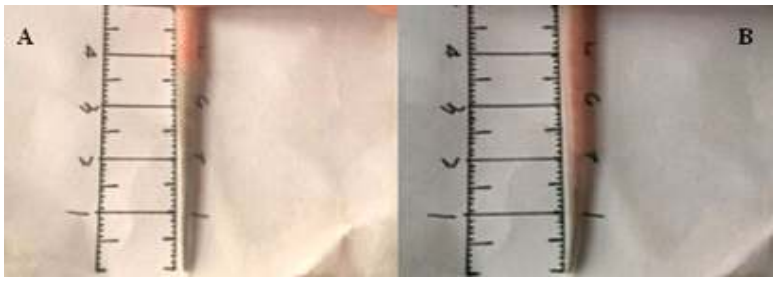


Fig. 6: Photographs of the infracted of mice after carrageenan was injected at 24 h. A, control group (carrageenan and saline water); B, sample group (carrageenan and crude enzyme)

signal to noise ratio and a value >4 is an essential prerequisite to fit the model well. Therefore, the proposed model can be used to navigate the design space.

The coefficients of the regression equation were calculated and the data were fitted to a second-order polynomial equation. The fibrinolytic enzyme production (Y) by *B. amyloliquefaciens* CGMCC 7380 can be expressed in terms of the following regression equation:

$$Y = -62062.12061 + 14327.63452A + 24619.46095B$$
$$- 884.86953AB - 1500.49409A^2 - 3258.5534B^2$$

where,
A = BBF concentration
B = Maltose concentration

Three-dimensional graph was generated for the combination of two independent factors (Fig. 5). The results showed that the global maximum in enzyme yield was confined within the experimental range. As a result, it gave the predicted maximum production with 5383.09 U/mL when the concentration of broad bean and maltose were at 38.1 and 32.6 g/L, respectively. Considering the cost and the operating convenience of the culture process, the optimal values of variables were determined as BBF 38.0 and maltose 32.0 g/L. To validate the predicted model, the tests were carried out at optimal medium in triplicate. After 48 h cultivation, the enzyme production was 4905.25±29.77 U/mL (n = 3), reaching 91.12% of predicted value.

Some studies have reported satisfactory optimization of fibrinolytic protease production from microbial sources using a statistical approach (Liu *et al*., 2005; Agrebi *et al*., 2009; Ku *et al*., 2009; Mahajan *et al*., 2010). However, few studies have reported that using broad bean as nitrogen substrate for the production of fibrinolytic enzyme. The effect of only addition of BBF (38.0 g/L) and maltose (32.0 g/L) on protease production in a medium was also carried out, the production of protease was 3747 U/mL, which higher than the wheat bran and cotton cake-from *Bacillus cereus* NS-2 (Bajaj *et al*., 2013) and *Mirabilis jalapa* tuber powders and yeast extract-from *Bacillus amyloliquefaciens* An6 (Agrebi *et al*., 2010). Therefore, this study provided a new method to produce fibrinolytic enzyme with high activity.

The thrombolytic activity of broad bean-fibrinolytic enzyme *in vitro* and *in vivo*: To address broad bean-fibrinolytic enzyme thrombolytic effects, we performed two experiments. Firstly, we performed an *in vitro* evaluation of broad-bean fibrinolytic enzyme and the protease could completely lyse artificial fibrin clot within 15 min. To further explore its thrombolytic activity *in vivo*, carrageenan-induced mouse tail thrombus model was used for the analysis. The tails of mice appeared auburn after carrageenan and saline treatment and the mean thrombosis length is 3.65±1.45 cm (Fig. 6A). In contrast, thrombus induced by carrageenan in mice tail was signigicantly reduced by broad bean-fibrinolytic enzyme (1.8±0.25 cm). From these results, it was evident that broad bean-fibrinolytic protease could be a useful thrombolytic agent (Fig. 6B).

CONCLUSION

This study described the production of fibrinolytic protease from a newly isolated *B. amyloliquefaciens* CGMCC 7380 strain cultivated in medium containing a new cheap fermentation substrate-broad bean and optimization the medium composition. The optimal concentrations for production of protease were (g/L): broad bean 38, maltose 32.0, $CaCl_2$ 0.2, K_2HPO_4 2.0, KH_2PO_4 2.0, $MgCl_2$ 0.5 and the maximum activity was 4905 U/mL. Moreover, the thrombolytic activity of broad bean-fibrinolytic enzyme *in vitro* and *in vivo* suggested that broad bean-fibrinolytic enzyme holds a potential to be functional food or thrombolytic drugs for thrombotic diseases. Further works should be done concerning the purification and characterization of this enzyme.

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