



# Optimization of fibrinolytic activity of *Solanum tuberosum* subtilisin-like protease (StSBTc-3) by response surface methodology

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## ABSTRACT

The aim of this study was to optimize *in vitro* conditions to enhance fibrinolytic activity of *Solanum tuberosum* subtilisin-like protease (StSBTc-3). The effects of StSBTc-3 concentration (0.2–5  $\mu$ M), pH value (6–10) and temperature (35–50 °C) on fibrinolytic activity were studied through response surface methodology (RSM). We obtained a model that predicts the response accurately. The relationship between enzyme concentration and fibrinolytic activity was linear, while the main effect from pH and temperature on the response was quadratic. From the RSM generated model the optimum pH was 8 and the optimum temperature was 43 °C, while higher concentrations of enzyme produce higher activities.

Under optimum conditions there were no statistically significant differences between the experimental responses and the ones predicted from the model. This model also predicts the activity under physiological conditions. These results confirm that StSBTc-3 is a good candidate to be considered for therapeutic uses. The generated model will be useful for biotechnological purposes.

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## 1. Introduction

Hemostasis is defined as the maintenance of fluidity of the blood and the protection of the body against bleeding. This is achieved by a complex mechanism involving platelets, vessel wall, coagulation and the fibrinolytic system [1]. Serine proteases are crucial for hemostasis. They are involved in several pathways of the process leading to clot formation and fibrinolysis [2].

Cardiovascular diseases are one of the major cause of death worldwide [3]. New drugs with anticoagulant and fibrinolytic effect have been approved for use and represent an enormous market opportunity for pharmaceutical companies [4,5]. The search for new drugs has led to the investigation of plant proteases and extracts as antiplatelet, anticoagulant or fibrinolytic compounds [6].

We have already reported the isolation and partial biochemical characterization of StSBTc-3, a subtilisin-like protease from *Solanum tuberosum* leaves [7]. Data obtained demonstrate that StSBTc-3 degrades all chains of human fibrinogen *in vitro* and inhibits platelet aggregation induced by collagen; therefore, StSBTc-3 has potential to be used in therapeutics for cardiovascular diseases [8].

Several factors like pH, temperature and enzyme concentration affect enzymatic activities. In a previous work we carried out a preliminary study on the effect that these variables have on the fibrinolytic activity [8]. However, this approach did not take into account the possible mutual interactions between variables.

Response surface methodology (RSM) was successfully utilized for optimization of several enzymatic reactions [9–13]. This technique involves an assortment of strategies to investigate ideal working conditions for a certain methodology. For quantitative variables RSM comprise fitting quadratic or linear functions of the independent variables to the response. Further actions are decided by characterizing of the model-generated surface [14]. The implementation of *rsm* package in R [15] facilitates classical response-surface methods. This type of analysis is described in Box et al., Khuri et al., Myers et al., Hunter et al., and Ryan et al. [16–20].

The aim of this work was to optimize the *in vitro* fibrinolytic activity of StSBTc-3, analyzing the effect of pH, temperature and enzyme concentration through RSM.

## 2. Material and methods

### 2.1. Enzyme purification

The StSBTc-3 enzyme was purified following the method previously developed by Fernández et al. [7]. The protease was extracted infiltrating potato leaves with buffer (15 ml of Tween 20;

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50 mM Tris–HCl (pH 8); 3.5% (w/v) NaCl; and 0.1% (v/v)  $\beta$ -mercaptoethanol) followed by centrifugation. StSBTC-3 was then purified from the washing fluid through ion-exchange chromatography. The eluted fraction with protease activity was purified again, through size exclusion chromatography to obtain the pure enzyme. The detailed protocol for purification and identification of this protease can be found elsewhere [8,7].

## 2.2. Protein concentration assay

Protein concentration was determined by quantification with bicinchoninic acid [21] with a commercial kit (PanReac Appli-Chem). The calibration curve was constructed by quantification with bovine serum albumin. For the assays, 25  $\mu$ l of sample was mixed with 200  $\mu$ l of bicinchoninic acid solution supplemented with  $\text{Cu}^{+2}$  according to the manufacturer's specifications. The reaction was incubated for 30 min at 37 °C. The absorbance (540 nm) was measured in a microplate reader (BioTek ELx800).

## 2.3. Response determination (fibrinogenolytic activity)

Responses were evaluated measuring the fibrinogenolytic activity of StSBTC-3. In order to do that the method utilized by Pepe et al. [8] was used. Solutions containing 40  $\mu$ g of human fibrinogen were incubated with StSBTC-3. Trichloroacetic acid (3% (v/v) final concentration) was added to the mixture to stop the reaction. The protein concentration of peptides remaining in solution after the precipitation was quantified using the protein concentration assay (Section 2.2). Enzymatic activity was defined as the amount of enzyme which increase absorbance in 0.001/min at 540 nm.

## 2.4. Design of experiments and data analysis

The *in vitro* fibrinogenolytic activity of StSBTC-3 was optimized through RSM. Assays were performed at different pH, temperatures and StSBTC-3 concentrations according to Table 2. Fibrinogenolytic activity was measured in order to select the optimum conditions of the enzyme (Section 2.3).

In this work a Box–Behnken design (BBD) was utilized to optimize the conditions through RSM analysis. The design consist in 12 experiments corresponding to a 3 factor BBD plus 3 experiments in the middle of the predictors variables domain.

The 3 variables studied were temperature, pH and enzyme concentration. The levels assigned for the predictors are shown in Table 1 and were selected using previous data [8].

The variables  $X_i$  were coded as  $x_i$ . The coded variables are defined according to Eq. (1) and they are dimensionless.

$$x_i = \frac{X_i - X_0}{\Delta X_i} \quad (1)$$

$\Delta X_i$  is the range for each predictor  $X_i$ ,  $X_0$  is the value of the real value at the central point,  $X_i$  is the real value of each independent variable and  $x_i$  is the corresponding coded value. In Table 1 are

**Table 1**  
Predictors variables and their values selected for the Box–Behnken design (BBD) for optimization of fibrinogenolytic activity of StSBTC-3.

Independent variable	Symbol		Levels		
	Uncoded	Coded	–1	0	1
Enzyme concentration ( $\mu$ M)	$X_1$	$x_1$	0.2	2.6	5.0
pH	$X_2$	$x_2$	6	8	10
Temperature (°C)	$X_3$	$x_3$	20	35	50

**Table 2**  
3 factors Box–Behnken design and responses obtained.

No.	$x_1$	$x_2$	$x_3$	Y
1	–1	–1	0	0.12
2	–1	1	0	0.15
3	–1	0	–1	0.17
4	–1	0	1	0.14
5	0	1	–1	0.35
6	0	1	1	0.37
7	0	–1	–1	0.25
8	0	–1	1	0.27
9	1	0	–1	0.45
10	1	0	1	0.75
11	1	–1	0	0.62
12	1	1	0	0.59
13	0	0	0	0.61
14	0	0	0	0.65
15	0	0	0	0.59

shown the predictors variables and the levels adopted for each one. The 15 runs were performed randomly to minimize bias. Two replications of each experiment were done and the average enzymatic activity was taken as the response, Y. The responses obtained are presented in Table 2.

A quadratic function was fitted to obtain the model that describes the relationship between the response (Y – fibrinogenolytic activity) and the 3 independent variables (coded). The equation below represent the model obtained:

$$Y = b_0 + b_1x_1 + b_2x_2 + b_3x_3 + b_{11}x_1^2 + b_{22}x_2^2 + b_{33}x_3^2 + b_{12}(x_1x_2) + b_{13}(x_1x_3) + b_{23}(x_2x_3) \quad (2)$$

where Y is the predicted response (predicted fibrinogenolytic activity),  $x_i$  are the coded independent variables,  $b_0$  the model constant,  $b_i$  the first order effect of  $x_i$ ,  $b_{ii}$  the quadratic effect of  $x_i$  and  $b_{ik}$  the interaction coefficient between  $x_i$  and  $x_k$ .

We used the implementation of *rsm* package [14] in R [15] to perform the regression analysis of the experimental data, the construction of the model and the graphical representation of the isoresponse curves. The significance of the coefficients was assessed through ANOVA. The fitting quality of the model was tested through the coefficient of determination  $R^2$ .

## 2.5. Verification and validation of the model

For verification of the model, StSBTC-3 fibrinogenolytic activity was measured under several conditions according to Section 2.3. The predicted data were compared with experimental measurements to assess the validity of the model.

## 3. Results and discussion

### 3.1. Fitting the models

We utilized RSM to perform a prediction model for optimize the *in vitro* fibrinogenolytic activity of the *Solanum tuberosum* serine protease (StSBTC-3). The experimental conditions and their responses from the BBD are presented in Table 2.

The independent variables and the response were analyzed in order to obtain the coefficients of Eq. (1). The values of these coefficients are presented in Table 3.

The regression equation that predicts the fibrinogenolytic activity (Y) is presented below:

$$Y = 6.17e^{-1} + 2.29e^{-1}x_1 + 2.50e^{-2}x_2 + 3.88e^{-2}x_3 - 8.96e^{-2}x_1^2 - 1.57e^{-1}x_2^2 - 1.50e^{-1}x_3^2 - 1.50e^{-2}(x_1x_2) + 8.25e^{-2}(x_1x_3) - 6.31e^{-18}(x_2x_3) \quad (3)$$

**Table 3**

Regression coefficients obtained for the RSM generated model. Signif. codes: 0 '\*\*\*'; 0.001 '\*\*'; 0.01 '\*'; 0.05 '.'; 0.1. Multiple R-squared: 0.979, Adjusted R-squared: 0.9411. F-statistic: 25.87 on 9 and 5 DF, p-value: 0.001132.

Term	Regression coefficient	Std. Error	t value	p value
$b_0$	$6.1667e^{-1}$	$3.0322e^{-2}$	20.3370	$5.317e^{-6}$ ***
$b_1$	$2.2875e^{-1}$	$1.8569e^{-2}$	12.3192	$6.240e^{-5}$ ***
$b_2$	$2.5000e^{-2}$	$1.8569e^{-2}$	1.3464	0.235999
$b_3$	$3.8750e^{-2}$	$1.8569e^{-2}$	2.0869	0.091263 .
$b_{12}$	$-1.5000e^{-2}$	$2.6260e^{-2}$	-0.5712	0.592572
$b_{13}$	$8.2500e^{-2}$	$2.6260e^{-2}$	3.1417	0.025618 *
$b_{23}$	$-6.3144e^{-18}$	$2.6260e^{-2}$	0.0000	1.000000
$b_{11}$	$-8.9583e^{-2}$	$2.7332e^{-2}$	-3.2776	0.022013 *
$b_{22}$	$-1.5708e^{-1}$	$2.7332e^{-2}$	-5.7472	0.002236 **
$b_{33}$	$-1.4958e^{-1}$	$2.7332e^{-2}$	-5.4728	0.002775 **

The plot of experimental responses vs those predicted from the model indicated a good fit (Fig. 1).

The results of analysis of variance (ANOVA) for the BBD are presented in Table 4.

Results obtained from the analysis of variance (Table 4) show that the pure quadratic (PQ) second-order term contribution is significant to the model, so the second order analysis is relevant. The two-way interaction (TWI) was not significant. Similar results were found for other proteases [22,13]. The coefficient of determination ( $R^2$ ) for the model fitted is 0.94 (Table 4). This implies that 94% of the variations could be explained by the fitted model.

### 3.2. Effect of independent variables on the response

The effects of enzyme concentration, pH and temperature were analyzed by RSM and the regression coefficients of the second order function are shown in Table 3. The contribution of the 3 variables studied was relevant to explain the responses obtained. Linear and quadratic contribution to the model were significant to explain the variance of the model, while two-way interactions were non significant (Table 4).

**Table 4**

Analysis of Variance Table for the RSM generated model. Multiple R-squared: 0.979, Adjusted R-squared: 0.9411. F-statistic: 25.87 on 9 and 5 DF, p-value: 0.001132. FO: first order; TWI: two-way interaction; PQ: pure quadratic.

Df	Sum	Sq	Mean Sq	F-value	Pr(> F)
FO( $x_1, x_2, x_3$ )	3	0.43563	0.145208	52.6435	0.0003324
TWI( $x_1, x_2, x_3$ )	3	0.02812	0.009375	3.3988	0.1105318
PQ( $x_1, x_2, x_3$ )	3	0.17843	0.059477	21.5627	0.0027360
Residuals	5	0.01379	0.002758		
Lack of fit	3	0.01192	0.003975	4.2589	0.1959881
Pure error	2	0.00187	0.000933		

The contribution of the linear term for enzyme concentration was significant ( $p < 0.0001$ ). Also, were significant the quadratic contribution for pH and temperature (Table 3).

The linear contribution for enzyme concentration ( $2.29e^{-1}$ ) is one magnitude order higher than the quadratic contribution ( $-8.96e^{-2}$ ), implying that the relationship between this variable and the response is approximately linear. These results agree with previous data obtained [8].

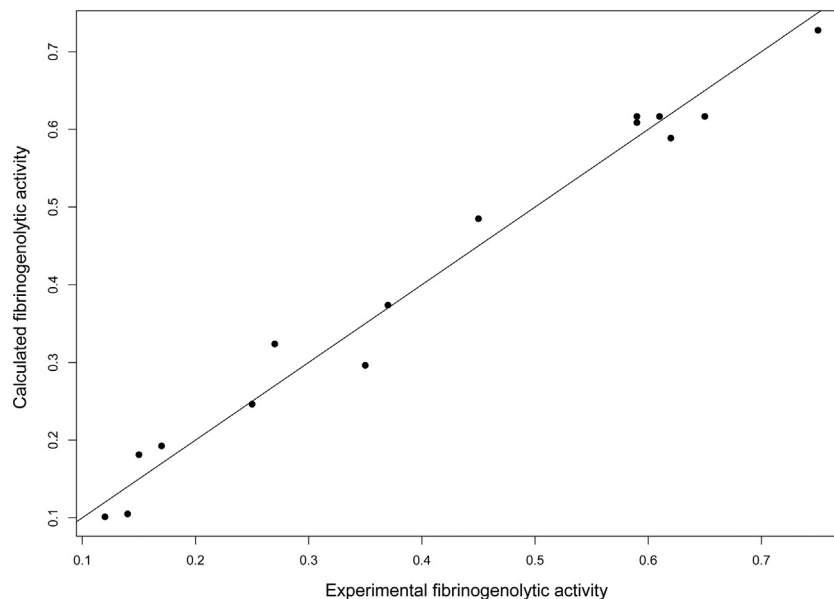
The quadratic coefficients for pH and temperature were higher than the linear ones (Table 3) implying the quadratic responses were relevant in these cases. The relation between variables and the response are shown in contour plots (Fig. 2) and response surfaces (Fig. 3).

Enzyme concentration shows a linear effect on the response, implying the fibrinogenolytic activity increase with the increment of StSBTC-3 concentration in a dose-dependent manner.

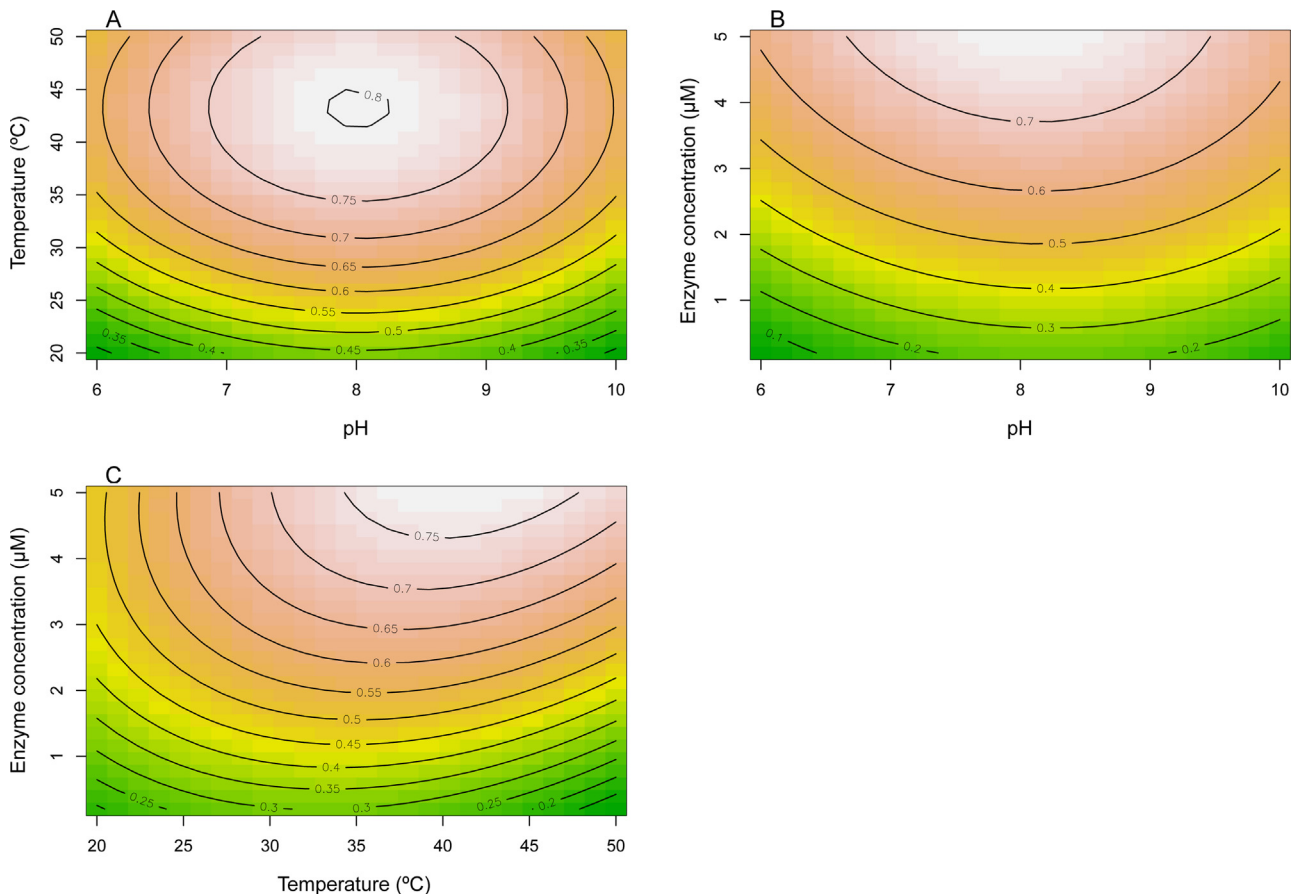
However, pH and temperature showed a quadratic effect on the response. Activity increase to about pH 8 and 40 °C and declines with further increase in both independent variables (Figs. 2 and 3).

### 3.3. Optimum conditions and verification of the model

Optimum conditions for testing fibrinogenolytic activity of StSBTC-3 were obtained from the model Eq. (3). Under optimum conditions of enzyme concentration 5  $\mu$ M, pH 8 and 43 °C, a maximum fibrinogenolytic activity of 0.62 was predicted. The model was further tested comparing the optimum response value



**Fig. 1.** Correlation of calculated response with experimental fibrinogenolytic activity.



**Fig. 2.** Contour maps showing the response for two variables at a time. The variable not shown in each plot was kept at the stationary point. (A) Temperature (°C) vs pH, slice at enzyme concentration of 5  $\mu\text{M}$ ; (B) enzyme concentration ( $\mu\text{M}$ ) vs pH, slice at 43.27 °C; (C) enzyme concentration ( $\mu\text{M}$ ) vs temperature (°C), slice at pH 8.

with independent experiments performed in the calculated optimum conditions. There were no statistically significant differences between experimental fibrinogenolytic activity (0.55) and the predicted value (0.62).

In previous work a preliminary characterization of StSTBc-3 varying one variable at a time was carried out without taken into account mutual interactions. An optimum pH (8) and temperature (40 °C) was found [8]. The results obtained in the present work agree with these data. This fact may be explained due to the non-significant mutual interaction of the variables. Each variable contribute independently to the response.

The optimum pH and temperature are in the same range as the ones obtained for other plant serine proteases [23,24]. This could imply that the fibrinogenolytic activity on human fibrinogen is due to the serine active site.

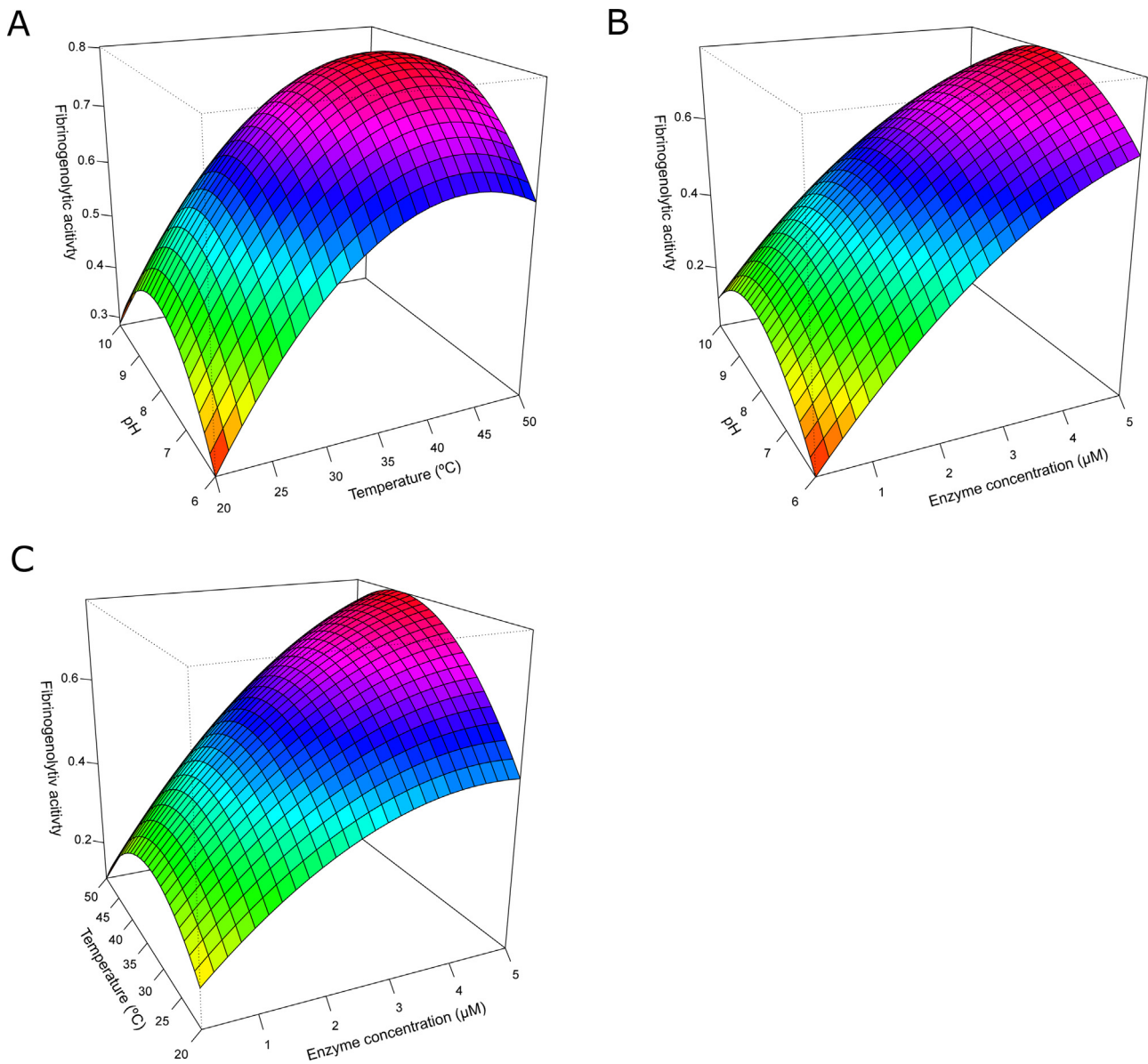
#### 4. Conclusions

Enzyme concentration, pH and temperature conditions were optimized with RSM in order to enhance the *in vitro* fibrinogenolytic activity of StSBTC-3.

From the RSM model optimum conditions for pH and temperature were 8 and 43 °C, respectively. The effect of enzyme

concentration on the response was linear, showing higher effect at higher concentrations. The activity corresponding to the optimum conditions were further confirmed by verification experiments. There are not significant difference between the experimental results obtained for fibrinogenolytic activity under optimum conditions and the predicted from the model.

This approach is better than the one used before because the Box-Behnken design utilized here reduce the number of experiments required to obtain statistically significant information. The RSM analysis implemented through the *rsm* package in R is a great tool to efficiently obtain the information required. It allowed us to obtain the optimum conditions for StSTBc-3 fibrinogenolytic activity. The model also predicts the response accurately in a wider variable's domain. This is useful for biotechnological purposes. Our future goal is to attach this enzyme onto a polymeric surface, and being able to predict the response will help us to predict the activity of the enzyme during the steps of the derivatization process. Through the surface plot analysis, we can determine the area of the variable's domain which correspond to higher activities. This allows as to predict the response under physiological conditions. From the model obtained, the activity under physiological conditions is still high enough to be considered for biomedical applications.



**Fig. 3.** Surfaces showing the response for two variables at a time. The variable not shown in each plot was kept at the stationary point. (A) Temperature ( $^{\circ}\text{C}$ ) vs pH, slice at enzyme concentration of  $5\ \mu\text{M}$ ; (B) enzyme concentration ( $\mu\text{M}$ ) vs pH, slice at  $43.27\ ^{\circ}\text{C}$ ; (C) enzyme concentration ( $\mu\text{M}$ ) vs temperature ( $^{\circ}\text{C}$ ), slice at pH 8.

### Author contributions

A.P. performed the assays, drew the figures and analyzed the data. F.R.T. help to perform biochemistry assays. M.G.G. designed the research and the manuscript. M.G.G. and A.P. wrote the manuscript. G.R.D. and F.R.T. revised the manuscript and checked English language.

### Conflicts of interest

The authors declare no conflict of interest.

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established researcher of CONICET and UNMDP; A.P. and F.R.T. are fellows of CONICET; G.R.D. is an established researcher of CIC.

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