Central Composite Design-based Optimization and Biological Characterization of Serralysin from a Novel Source by Solid State Fermentation

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Serralysin is well known to exhibit anti-inflammatory and fibrinolytic properties. The current research designed a cost-effective serralysin production medium from *Streptomyces hydrogenans* var. MGS13 with the aid of solid state fermentation. Four pre-screened factors, namely horse gram flour concentration, inoculum size, initial moisture content and soya bean meal were modeled by central composite design for optimizing in order to predict their influence on serralysin production. Analysis of variance results showed a high coefficient of determination (R^2) value of 0.9611, ensuring a satisfactory adjustment of the quadratic model with the experimental data and F value 26.45 (p value of <0.0001) indicated that the model was significant. The design of experiment assisted production process enhanced 1.3 fold productivity at the best possible conditions consisting 5.0 g of horse gram flour, 1.2 ml of inoculum (1×10^6 CFU/ml), 44 % of initial moisture content and soya bean meal 1.0 % w/w. Besides this study, the *in vitro* fibrinolytic and anti-inflammatory activities were carried with purified serralysin of *Streptomyces hydrogenans* var. MGS13. The results revealed that the purified enzyme exhibited fibrinolytic and anti-inflammatory activity in a dose dependent manner. Further one-way analysis of variance and Dunnett's multiple comparisons statistically justify the data p<0.05 in both activities.

Key words: Horse gram flour, *Streptomyces hydrogenans* var. MGS13, central composite design, solid state fermentation, fibrinolytic activity, anti-inflammatory activity

"Healing power of nature has been well-known since ages, as the source of all medicine has been lying within Mother Nature". Particularly, enzymes occur universally in wide variety of sources like microbes, plants and animals. Microbes are appeared as promising sources for production of peptidases over plants and animals. Peptidases such as trypsin, chymotrypsin and serralysin (also called as serratiopeptidase and serrapeptase) belong to the class of hydrolases and used clinically as anti-inflammatory agents[1,2]. Among these peptidases, serralysin has been effectively employed in disorders such as inflammation, pain and atherosclerosis[3,4]. Anti-inflammatory mechanism of serralysin involves the hydrolytic cleavage of inflammatory mediators such as bradykinin, serotonin and histamine[5]. Fibrinolytic mechanism of serralysin is due to enhancing plasmin activity by antagonizing the plasmin inactivators[6]. Serratiopeptidase (EC 3.4.24.40) comes under metzincin class of proteases as per the MEROPS database[7] which was initially produced from *Serratia marcescens* furthermore, it was reported from other bacterial strains such as *Pseudomonas aeruginosa, Proteus mirabilis, Escherichia freundii*[8], *Xenorabdus*[9,10], *Deinococcus radiodurans*[11] and *Bacillus subtilis*[12].

Till date serralysin produced from an opportunistic pathogen is available in market which is being associated with unwanted effects such as lung and corneal damage[13] therefore, it arises an increased need of novel microbial sources for bio-better serralysin production. Even though surplus numbers of reports are accessible for

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serralysin production from bacterial strains there is meager reports available pertaining serralysin production from *Strepomyces* sp. In this regard *Strepomyces*. sp gained much attention due to ability for the bioactive metabolites (proteases, cellulose, keratinase, and fibrinolytic enzymes) production[14,15]. Previously, serralysin producing isolate was screened and identified as *Streptomyces hydrogenans* (*S. hydrogenans*) var. MGS13 in Pharmaceutical Biotechnology laboratory[16], further produced the same enzyme by Sub-Merged Fermentation (SMF)[17]. This investigation chooses, Solid State Fermentation (SSF) technique for serralysin production from the same isolate because, the preferred technique not only resembles as a natural habitat for growth of organism but also reduces the production cost of metabolite. In this regard an increasing emphasis is being laid on the microbial proteases production by SSF where cheap agro residues were employed to produce worthy bioactive metabolites[18,19].

In the previous study, authors have reported serralysin production using this novel isolate (*S. hydrogenans*), chromatographically purified, characterized and structure of the enzyme was also predicted further confirmed that the produced enzyme is a serralysin like alkaline metalloprotease[20]. The optimization of growth medium components exerts a huge impact on cell growth and yield of bioactive metabolites. The One Factor At a Time (OFAT) approach is frequently used classical approach for designing the initial medium composition, but this strategy does not identify the interaction effects among the variables. To overcome this drawback, statistical method (Response Surface Method (RSM)) has been efficiently employed for achieving maximum productivity[21]. Hence in the present investigation, Central Composite Design (CCD) based RSM model was opted to enhance the serralysin yield by optimizing the cultivation medium of *S. hydrogenans* var. MGS13. Further, the biological activities of purified serralysin of *S. hydrogenans* var. MGS13 such as *in vitro* fibrinolytic and *in vitro* anti-inflammatory activity were also investigated in detail.

MATERIALS AND METHODS

Source of the microorganism:

The novel strain was screened and isolated from Koringa mangrove soil which was used in previous study for the production of serralysin by SMF in Pharmaceutical Biotechnology division, Andhra University. For identification the strain was sent to Institute of Microbial Technology, Chandigarh showed 99.91 % identity to homologous fragments of *S. hydrogenans* hence, it was identified as *S. hydrogenans* var. MGS13[16]. The same strain was selected in the present study and deposited in National Collection of Industrial Microorganisms, Pune India with deposit number 5745.

Preparation of seed culture:

The seed culture for fermentation was prepared by scrapping the spore surface of 7 d old well sporulated cultures with 10 ml sterile distilled water, then the cell suspension was diluted and made up to 50 ml which was used as inoculum for execution of 30 treatment combinations. The cell density of cell suspension was determined in terms of CFU/ml by using colony counter which was found to be 1×10^6 CFU/ml and the same was maintained for all experiments based on the earlier findings of several other researchers[22].

Production of serralysin by SSF:

Horse gram flour (5g each) of average particle size 425 microns were placed in 250 ml Erlenmeyer flasks (B.S.S 25 American Standard Test Sieve Series (ASTM) sieve passed and B.S.S 60 ASTM sieve retained particles) hydrated to initial moisture content 40 % v/w and 1 % w/w of soya bean meal was added. The initial medium pH 6.5-7.0 was noticed and all the contents were vigorously mixed and autoclaved at 121° for 20 min, cooled and inoculated with 1 ml of cell suspension of *S. hydrogenans* var. MGS13 carrying 1×10^6 CFU/ ml and incubated at 28° for 96 h. All these fermentation parameters were selected based on the results obtained in the classical method (OFAT)[23] and the trails were done in triplicates. After incubation, the culture contents were extracted with sodium borate buffer (pH 9.0) (50 ml/ flask) by squeezing through a wet sterile cloth and centrifuged at 8000 ×g at 4° for 20 min. The cell free supernatant was collected and used for the determination of serralysin assay.

Quantitative assessment of serralysin assay:

The quantitative assay for the evaluation of serralysin activity was done as per Indian Pharmacopoeia 2010[24]. One serralysin unit is defined as the amount of enzyme required to liberate 1 µmol of free tyrosine

per ml per min under the specified experimental conditions.

Optimization process of serralysin with the aid of CCD based RSM design:

In order to achieve sample yield of serralysin, individual as well as interaction effects[25] of variables were determined with the aid of central composite design. In the proposed experimental model horse gram flour (substrate) concentration, inoculum size, initial moisture content and soya bean meal were selected from the OFAT study[23] and these selected variables are considered for further optimization by implementing RSM based CCD in order to ascertain the individual and interaction effects on serralysin production. The code for each independent variable and their ranges in the design are given in Table 1. The CCD design studying 4 significant variables was generated using design Expert 12.0 software (Stat-Ease Inc. Minneapolis, USA) for execution of 30 experiments.

For four-factor system, the regression model equation (Eq. (1)) is derived as below.

$$Y=\beta_0+\beta_1A+\beta_2B+\beta_3C+\beta_4D+\beta_{11}A^2+\beta_{22}B^2+\beta_{33}C^2+\beta_{44}D^2+\beta_{12}AB+\beta_{13}AC+\beta_{14}AD+\beta_{23}BC+\beta_{24}BD+\beta_{34}CD \quad (1)$$

Where Y: Predicted response; β_0: Intercept, A: Horse gram flour concentration, B: Inoculum level, C: Initial moisture content, D: Soya bean meal, $\beta1$, $\beta2$, $\beta3$, $\beta4$: linear coefficients; β_{11}, β_{22}, β_{33}, β_{44} are the squared coefficients; β_{12}, β_{13}, β_{14}, β_{23}, β_{24}, β_{34}: interaction coefficients.

Each independent variable was separated into three levels, namely positive level, middle level or zero level and negative level. Every level was included in the run matrix for the study on effect of various independent variables on serralysin production by *S. hydrogenans* var. MGS13. Here, each experiment was done in three sets.

Statistical analysis and validation of experimental model:

Analysis of Variance (ANOVA) was employed for analyzing the data to identify the most effective independent variables and its interaction effects for maximum serralysin production. The experimental model was validated with respect to all variables within the design space. The optimized combinations were experimented each in triplicate and the observed mean response was compared with the predicted value.

Purification and characterization of serralysin:

Extracellualr enzyme of *S. hydrogenans* var. MGS13 was extracted from fermented bran, which was further precipitated and purified by gel filteration method using Sephadex G-100[20].

Native Polyacrylamide Gel Electrophoresis (PAGE) (non-denaturing gel electrophoresis):

As a trail native PAGE was done according to the method of Laemmli *et al.*[26] with various percentages of polyacrylamide resolving gel i.e., 10, 12 and 15 % to know the exact sieving gel percentage of the crude enzyme under non-denaturing conditions. The sample was prepared in Sodium Dodecyl Sulfate (SDS) free buffer, under non-reducing (without 2-βmercaptoethanol) and non-denaturing conditions (without heating) and the stacking and separating gel was also prepared without using SDS.

SDS-PAGE:

The purity of enzyme was ascertained by SDS PAGE according to Laemmli *et al.*[26] using 15 % polyacrylamide resolving gel.

Evaluation of biological activities of serralysin:

The purified serralysin has been examined for its biological properties by performing *in vitro* fibrinolytic and anti-inflammatory activities.

TABLE 1: EXPERIMENTAL VARIABLES AND THEIR LEVELS USED IN CCD

Variables	Range of levels				
	-2	-1	0	1	2
Horse gram flour concentration (g)	3	4	5	6	7
Inoculum size (ml)	0.5	0.75	1	1.25	1.5
Initial moisture content (% v/w)	30	35	40	45	50
Soya bean meal (% w/w)	0.5	0.75	1	1.25	1.5

***In vitro* fibrinolytic activity of purified serralysin:**

The fibrinolytic activity of the purified serralysin was assessed by *in vitro* 'fibrin clot lytic method' according to Rajput *et al.*[27] with slight modifications. The preparation of fibrin clot was done as per Sigma-Aldrich procedure by cross-linking of fibrinogen with thrombin. Both fibrinogen and thrombin solutions were prepared in 0.9 % NaCl. About 5 ml aliquot of fibrinogen solution (10 mg/ ml in saline) was added to 0.5 ml of 50 U/ml solution of thrombin in saline. Then the solution was mixed thoroughly and permitted to stand for 1 h incubation at 37° to ensure the polymerization of fibrin clot. Fibrinolytic activity was performed with different concentrations of purified serralysin (250, 500, 750, 1000 U/500 µl) and commercial serratiopeptidase (100, 200, 300, 400 and 500 U/500 µl). All test tubes containing fibrin clot were labeled properly and thereafter, 500 µl of each sample was added separately into each tube and allowed to stand 8 h incubation at 37°. As a control, borate buffer pH 9.0 (500 µl) was added to fibrin clot and incubated under same experimental conditions. After incubation, volume of fluid obtained was measured to perceive the change in volume after of lysis fibrin clot. The percentage fibrinolysis was calculated by measuring the difference obtained in volume of fluid taken before and after fibrin clot lysis. All trials were repeated in triplicate. The software Graph Pad Prism version 5.0 has been used to analyze the obtained data. All values are expressed as mean±standard error of mean. Data were analyzed by one-way ANOVA and difference between means was assessed by Dunnett's multiple comparison. $p<0.05$ was considered statistically significant.

***In vitro* anti-inflammatory activity of purified serralysin:**

The anti-inflammatory activity of purified serralysin enzyme was assessed by *in vitro* Human Red Blood Cell (HRBC) membrane stabilization method according to Shinde *et al.*[28] with minor modifications. Sample solutions of purified serralysin enzyme (100, 200, 300, 400 and 500 IU/ml), commercial serratiopeptidase (100, 200 IU/ml) and diclofenac (200 µg/ml) were prepared by dissolving the corresponding stock solutions made up of hypotonic and isotonic buffers.

The erythrocyte suspension was prepared by drawing fresh blood sample (3 ml) from healthy volunteer who is not any drug therapy for the past 2 w and transferred into the heparinized tubes to prevent clotting and centrifuged at 3000 rpm for 10 min at 4°. After centrifugation, supernatant was measured, discarded and an equivalent volume of normal saline solution was used to dissolve red blood pellet. The acquired quantity of red blood cell pellet was measured and re-constituted as a 40 % v/v cell suspension with isotonic buffer (10 mM sodium phosphate buffer, pH 7.4). Haemolysis was induced by dissolving the samples under test in hypotonic solution (distilled water) and isotonic solution respectively. About 5 ml of hypotonic solution containing graded concentration of enzyme solutions (100, 200, 300, 400 and 500 U/ml) were set into 2 pairs (per concentration) of small test tubes. About 5 ml of isotonic solution also containing graded concentration of enzyme solutions (100, 200, 300, 400 and 500 IU/ml) were also set into 2 pairs (per concentration) of the small test tubes.

The control tubes were containing 5 ml of hypotonic solution and 5 ml of graded dose (100 and 200 IU/ml) of commercial serratiopeptidase and diclofenac (200 µg/ml) were prepared with hypo and isotonic solutions respectively. About 0.1 ml of erythrocytic suspension was incorporated to the mixture of each of test tube and mixed gently. Further the sample mixtures were incubated for 1 h at 37° and centrifuged for 10 min at 3000 rpm at 4°. The supernatant was collected further absorbance (Optical Density (OD)) of the hemoglobin content of supernatant was valued at 540 nm using visible spectrophotometer. The haemolysis produced in the presence of hypotonic solution was considered as 100 %. The inhibition percentage of haemolysis was estimated by using the given formula.

Percentage of inhibition of haemolysis=$1-(OD_2-OD_1/OD_3-OD_1)\times100$

Where OD_1=Absorbance of the test sample in isotonic solution; OD_2=Absorbance of the test sample in hypotonic solution; OD_3=Absorbance of the control sample in hypotonic solution

The software Graph Pad Prism version 5.0 has been used to analyze the obtained data. All values are expressed as mean±standard deviation. Data were analyzed by using one-way ANOVA and difference between means was assessed by Dunnett's multiple comparison. $p<0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

Serralysin has been extensively employed in the treatment of inflammation and atherosclerotic disease (by dissolving fibrin clot)[2]. RSM based CCD design matrix was applied to identify the best possible level of individual parameters and their combined effects on serralysin production from *S. hydrogenans* var. MGS13 with four process variables namely horse gram flour concentration, initial moisture content, inoculum level and soya bean meal by employing SSF. Based on the results of traditional OVAT method[23], the process variables were selected and considered as center point in CCD design. Identification of appropriate solid substrate is a vital step in SSF process. So the preliminary screening was conducted by using various particle sizes (ranging from 850-250) of various solid substrates (Rice bran, wheat bran, green gram husk, black gram husk, rice flour and horse gram flour) were used. Among them 425 microns of horse gram flour was found to be suitable for serralysin production from *S. hydrogenans* var. MGS13. There were no reports available on production of serralysin using horse gram flour as a solid substrate in the SSF. Although horse gram is an excellent source of proteins but its consumption is very less due to unacceptable taste and flavor of cooked products, so it remained as an underutilized crop. Thus utilizing this crop residue as a nutrient source will not only support the growth of organism but also reduces the overall production cost. The CCD based RSM model was designed with the result of 30 treatments (runs) then actual and predicted response of serralysin activity (U/gds) are presented in Table 2. The significance of this model was checked with ANOVA using F test and multiple regression analysis was applied to obtain a second order polynomial equation (1). Depending on the selected variable combination, serralysin activity was varied over a range (from 7.24 to 93.78). After regression analysis, the regression equation for serralysin production in terms of coded variable and actual variables was obtained as presented below in equation (2).

TABLE 2: EXPERIMENTAL AND PREDICTED VALUES OF SERRALYSIN YIELD RECORDED IN THE EXPERIMENTAL SETUP OF RSM

Std	Runs	A: Horse gram flour concentration (g)	B: Inoculum size (ml)	C: Initial moisture content (%v/w)	D: Soya bean meal (% w/w)	Serratiopeptidaseactivity (U/gds)	
						Experimental	Predicted
12	1	6	1.25	35	1.25	59.21 ±0.02	59.47
15	2	4	1.25	45	1.25	68.98±0.02	73.4
1	3	4	0.75	35	0.75	21.31±0.03	23.26
5	4	4	0.75	45	0.75	65.90±0.05	62.9
24	5	5	1	40	1.5	55.29±0.01	59.2
3	6	4	1.25	35	0.75	30.33±0.01	31.35
25	7	5	1	40	1	78.85±0.00	78.85
23	8	5	1	40	0.5	60.24±0.03	58.88
2	9	6	0.75	35	0.75	13.46±0.03	6.3
7	10	4	1.25	45	0.75	78.01±0.08	79.6
27	11	5	1	40	1	78.85±0.00	78.85
26	12	5	1	40	1	78.85±0.00	78.85
29	13	5	1	40	1	78.85±0.00	78.85
8	14	6	1.25	45	0.75	89.93±0.09	78.12
17	15	3	1	40	1	30.33±0.02	30.1
19	16	5	0.5	40	1	25.22±0.03	29.1
20	17	5	1.5	40	1	93.78±0.05	92.45
18	18	7	1	40	1	7.24±0.01	10.03
9	19	4	0.75	35	1.25	22.34±0.11	31.41
4	20	6	1.25	35	0.75	18.24±0.51	30.09
11	21	4	1.25	35	1.25	67.88±0.02	62.38

22	22	5	1	50	1	55.45±0.02	64.34
10	23	6	0.75	35	1.25	14.26±0.02	12.82
21	24	5	1	30	1	20.22±0.05	13.88
30	25	5	1	40	1	78.85±0.00	78.85
16	26	6	1.25	45	1.25	72.09±0.01	70.29
28	27	5	1	40	1	78.85±0.00	78.85
13	28	4	0.75	45	1.25	45.54±0.00	33.84
14	29	6	0.75	45	1.25	18.78±0.09	15.03
6	30	6	0.75	45	0.75	40.09±0.09	45.74

Serralysin activity= $+78.85\times A-5.02\times A+15.84\times B+12.61\times C+0.0796\times D+3.92\times AB-0.0531\times AC-0.4106\times AD+2.15\times BC+5.72\times BD-9.31\times CD-14.70\times A^2-4.52\times B^2-9.93\times C^2-4.9\times D^2$ (2)

Where Y: Response (Serralysin activity in (U/gds), A: Horse gram flour concentration (g); B: Inoculum size (ml); C: Initial moisture content (% v/w); D: Soya bean meal (% w/w) respectively.

The ANOVA of the regression model expressed the significance of the parameters with effect of serralysin production as evident from the high F value 26.45 and low p value of <0.0001 (Table 3). There is only 0.01 % probability of the model F-value could happen due to noise. The values of 'Prob>F' less than 0.0500 implies that the model terms such as linear terms horse gram flour concentration (A); inoculum size (B); initial moisture content (C); interactive terms BD (inoculum size and soya bean meal); CD (initial moisture content and soya bean meal) and the quadratic terms horse gram flour concentration2 ((A^2); inoculum size2 (B^2); initial moisture content2 (C^2) and soya bean meal2 (D^2) were found to be significant for serralysin production. The predicted and experimental values plot illustrated that actual values were nearer to the straight line (fig. 1) indicating that experimental serralysin activity (93.78) under optimized condition was in strong agreement with predicted serralysin activity (92.45).

The coefficient of determination (R2) was 0.9611 indicated that 96.11 % variability in the response data could be elucidated by this model. The R2 value 0.9611 which is closer to 1 depicted that the model is statistically good. The predicted R2 of 0.7757 is in reasonable agreement with the adjusted R2 of 0.9247 because the difference is less than 0.2. An "adequate precision" measures the signal to noise ratio and the desirable value should be more than 4. A ratio16.390 indicates that the proposed model is significant. The ANOVA results depicted that the horse gram flour concentration had a significant influence on serralysin production. An optimum yield of serralysin was obtained with 5.0 g of horse gram flour. The concentration of solid substrate plays an essential role in enzyme production because it provides essential nutrients, growth factors to microbes and also minimizes the manufacturing cost[19,29,30]. Like other solid substrates horse gram flour also supports same substrate concentration for SSF. The ANOVA results depicted that inoculum size had a remarkable influence on serralysin production and maximum yield of serralysin was achieved with 1.2 ml of inoculums *S. hydrogenans* var. MGS13. Beyond 1.2 ml of inoculum size a decrease of enzyme yield was observed which could be due to exhaustion of nutrients in the fermented medium whereas lower inoculum level of *S. hydrogenans* var. MGS13 required a more time for its growth. Numerous studies have detailed that optimum size of inoculum is crucial for achieving maximum biomass and yield of metabolite[19,31]. Depending up on the type of microorganism, and concentration of substrate appropriate size of inoculums was varied *Bacillus* sp. showed maximum protease production with 20 % v/ w inoculum[32-34] whereas maximum yield of rhamnolipid was achieved with 2.5 ml inoculum of Serratia rhubidaea[29]. The ANOVA results revealed initial moisture content had a significant influence on serralysin production and optimum serralysin yield was achieved at 44 % of initial moisture content whereas at lower and higher moisture contents resulted in reduction of serralysin production. This may be because of less solubility of nutrients in solid substrate medium at low moisture level; while at higher moisture levels low protease yield was noticed because of stickiness and decreased porosity of solid substrate[19,34,35]. The 3D-response surface plot (fig. 2a) obtained as a function of horse gram flour concentration versus inoculum size, where maximum serralysin yield (90 U/gds) was noticed with 5.1 g horse gram flour concentration and 1.2 ml inoculum.

The 3D-response surface plot (fig. 2b) obtained as a function of inoculum size versus initial moisture content, where maximum serralysin yield (95 U/gds) was observed with 1.2 ml inoculum size and 44 % v/w initial moisture content. The 3D response plot (fig. 2c) obtained as a function of inoculum size *vs.* soya bean meal where maximum serralysin yield by 91 U/gds was noticed with inoculum size 1.2 ml and soya bean meals 1.2 % w/w further increasing or decreasing their levels resulted in decrease in serralysin yield. The 3D-response surface plot (fig. 2d) depicted the interaction between horse gram flour concentration and soya bean meal where no improvement of serralysin yield was noticed with this interaction. The 3D response plot (fig. 2e) acquired as a function of horse gram flour concentration *vs.* initial moisture content and (fig. 2f) acquired as a function of initial moisture content versus soya bean meal where serralysin yield was improved with increasing initial moisture content up to 44 %, further increasing its level resulted in gradual decrease in serralysin yield.

The developed design was validated by performing the experiment with predicted optimized parameters such as horse gram flour (5.0 g), inoculum (1.2 ml), initial moisture content (44 % v/ w), soya bean meal (1.0 % w/w). Under these optimized conditions the experimental serralysin activity (94 U/gds) was determined to be in quite close the predicted serralysin activity (93 U/gds) which confirmed that the developed model was reliable. This study resulted in 1.3 fold increase on serralysin production (94 U/gds) than initial level (78 U/gds).

The extracellular supernatant of enzyme was extracted from fermented bran, precipitated, further purified by gel filtration using Sephadex G100 and the process was clearly reported by authors in the previous reports[20]. An appropriate resolving gel percentage is required for good mobility and separation of sample. So, as a trail in the present study Native PAGE of crude enzyme was carried with various percentage of resolving gel (10, 12 and 15 %). The observed multiple bands in native PAGE represents that 15 % resolving gel was found to be appropriate sieving gel percentage for the crude enzyme (fig. 3a). Further the purity of the enzyme was verified by performing a SDS PAGE with 15 % resolving gel where a single band was observed (fig. 3b) which indicates that the enzyme is homogenous in nature. Previously we have reported the characterization studies, peptide mapping analysis and bioinformatics studies based on that we confirmed that this purified enzyme is an alkaline metalloprotease[20].

TABLE 3: ANALYSIS OF VARIANCE FOR CCD RESULTS OF SERRALYSIN PRODUCTION

Source of variance		Sum of Squares	df	Mean Square	F-value	p-value
Model		20459.81	14	1461.42	26.45	<0.0001 significant
Linear effects	A-Horse gram flour concentration	604.11	1	604.11	10.93	0.0048
	B-Inoculum size	6020.15	1	6020.15	108.95	<0.0001
	C-Initial moisture content	3819.07	1	3819.07	69.11	<0.0001
	D-Soya bean meal	0.152	1	0.152	0.0028	0.9589
Interaction effects	AB	246.25	1	246.25	4.46	0.052
	AC	0.0452	1	0.0452	0.0008	0.9776
	AD	2.7	1	2.7	0.0488	0.8281
	BC	74	1	74	1.34	0.2653
	BD	523.15	1	523.15	9.47	0.0077
	CD	1385.51	1	1385.51	25.07	0.0002
Quadratic effects	A2	5923.26	1	5923.26	107.19	<0.0001
	B2	559.52	1	559.52	10.13	0.0062
	C2	2706.12	1	2706.12	48.97	<0.0001
	D2	672.15	1	672.15	12.16	0.0033
Residual		828.86	15	55.26		
Lack of Fit		828.86	10	82.89		
Pure Error		0	5	0		
Corrected Total		21288.67	29			

Note: *The model F-value 26.45 implies the model is significant; A: Horse gram flour concentration B: Inoculum size; C: Initial moisture content and D: Soya bean meal

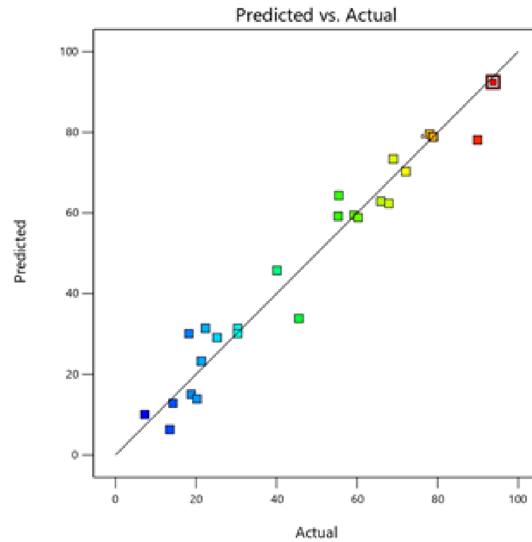

Fig. 1: Residual diagnostics of contour surface of the quadratic model by predicted *vs.* actual serralysin production of *S. hydrogenans* var. MGS13

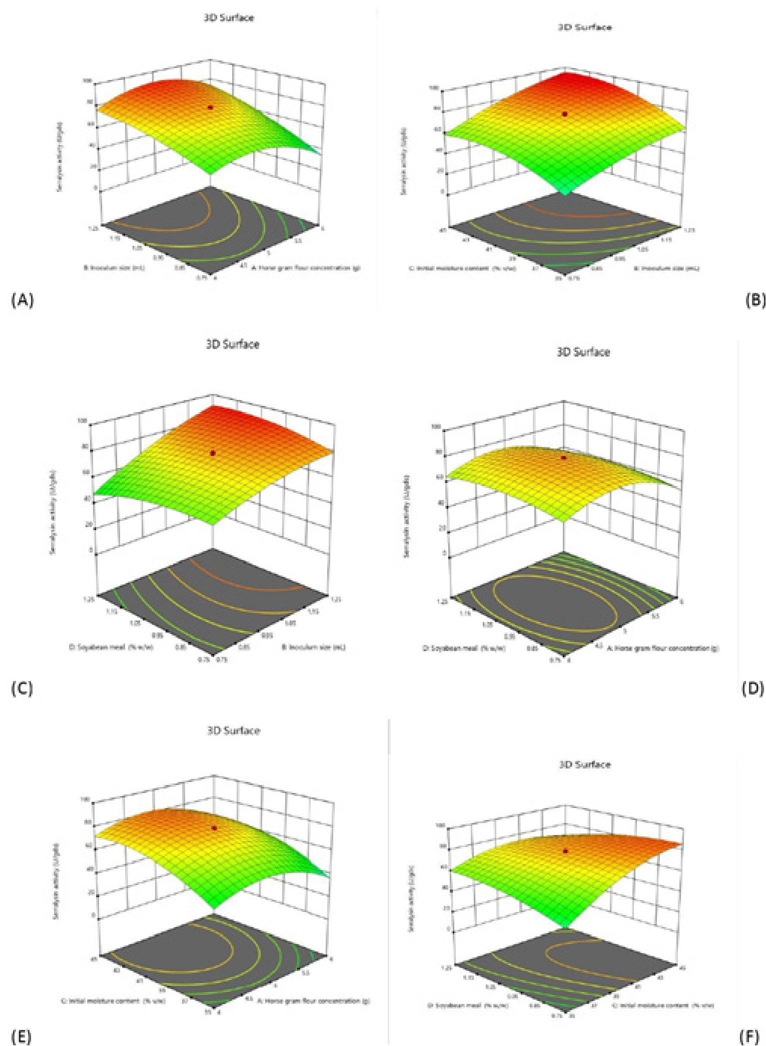

Fig. 2: 3D-response surface graph for serralysin production showing interaction between two variables
Note: (a): The interaction between horse gram flour concentration (A) and inoculum (B); (b): The interaction between inoculum size (B) and initial moisture content (C); (c): The interaction between inoculum size (B) and soybean meal (D); (d): The interaction between horse gram flour concentration (A) and soybean meal (D); (e): The interaction between horse gram flour concentration (A) and initial moisture content (C) and (f): Interaction between initial moisture content (C) and soybean meal (D)

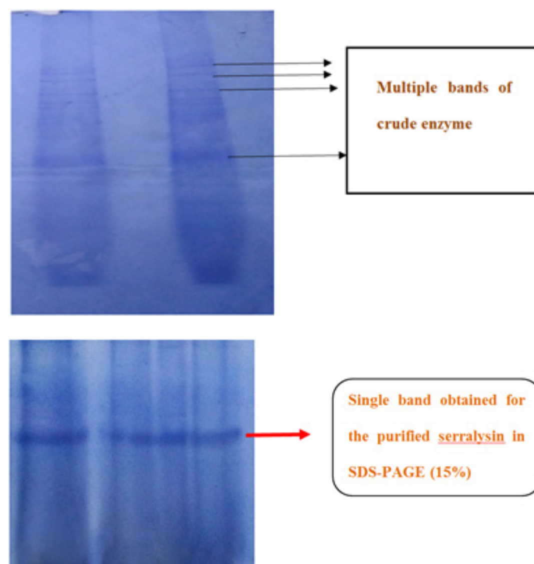

Fig. 3: (A): Native PAGE of crude enzyme and (B): SDS-PAGE of purified sample

In vitro fibrinolytic and anti-inflammatory activities of purified serralysin were further performed in order to verify the biological properties of serratiopeptidase. Fibrin clot lytic method was employed for assessment of fibrinolytic activity using various concentrations of standard (commercial serratiopeptidase) as positive control and purified serralysin of *S. hydrogenans* var. MGS13 as test and buffer as negative control. The results (Table 4) were validated by demonstrating a dose dependent graph (fig. 4) using Prism-software (Version 5.0). The investigation suggested that both purified serralysin (250-1000 IU/500 µl) and commercial serratiopeptidase (100-500 IU/500 µl) showed dose dependent fibrinolysis because the activity was increased with the increasing concentrations. Clots were treated with borate buffer pH 9.0 showed negligible clot lysis. The mean difference in percentage clot lysis between each dose of purified serralysin enzyme, commercial serratiopeptidase enzyme and buffer (control) was significant ($p<0.05$). Further half maximal inhibitory concentration (IC_{50}) values of purified serralysin (fig. 5a) and commercial serratiopeptidase (fig. 5b) for fibrinolysis were calculated by plotting log dose *vs*. fibrinolysis respectively and found to be 403.1 and 172.2 IU respectively. After incubation of 8 h, the purified serralysin showed 81 % fibrinolysis at a concentration of 1000 IU/500 µl when compared to commercial serratiopeptidase same activity was exhibited at concentration of 500 IU/500 µl, further the lysis of clot was visually observed during incubation. Serrapeptase is a plasmin like protease which could directly dissolves fibrin clot[36], hence it is the more advantageous over plasminogen activators such as urokinase, streptokinase and tissue plasminogen activators (t-PA) where the enzyme converts the blood plasminogen to clot dissolving plasmin. From the present research, it is denoted, that the purified serralysin has potent fibrinolytic activity like commercial serratiopeptidase, which conforms that purified serralysin of *S. hydrogenans* var. MGS13 shares serralysin kind of nature. As mentioned above some reports on serralysin like alkaline metalloprotease from various sources such as *Serratia* sp. and *Xenorhabdus* also exhibited strong fibrinolytic property[36,10].

In vitro anti-inflammatory activity was studied by measuring the percentage inhibition of hemolysis for all concentrations of purified serralysin enzyme, commercial serratiopeptidase and diclofenac (Table 5). Various concentrations (100-500 IU/ml) of purified serralysin of *S. hydrogenans* var. MGS13 exhibited varying percentage inhibition of haemolysis were 28, 38, 42, 48 and 54 (fig. 6). Control groups treated with diclofenac (200 µg/ml) and commercial serratiopeptidase (100, 200 IU/ml) showed 67, 34 and 44 percentage inhibition of haemolysis respectively. A concentration dependent anti-inflammatory activity was noticed with both purified enzyme and commercial serratiopeptidase. The data revealed that purified serralysin significantly (p value <0.05) inhibited hemolysis induced by hypotonic solution (water). The percentage inhibition of haemolysis by purified serralysin enzyme was 0.85 times when compared to the commercial serratiopeptidase.

TABLE 4: PERCENTAGE OF FIBRINOLYSIS EXHIBITED BY VARIOUS CONCENTRATIONS OF PURIFIED SERRALYSIN AND COMMERCIAL SERRATIOPEPTIDASE

Dose (IU/500 µl)	Percentage fibrinolysis purified serralysin	Commercial serratiopeptidase
100	-	35.55±0.89***
200	-	52.47±0.93***
250	30.50±0.80***	-
300	-	64.87±0.86***
400	-	74.65±1.50***
500	59.55±0.89***	81.77±0.86***
750	75.54±0.88***	-
1000	81.89±0.86***	-
Control	8.7±0.03***	-

Note: Results are expressed as mean±standard error of mean; n=3 in each group comparison made with control group. Data was analysed by one way ANOVA followed by Dunnett's test; ***p<0.005 when compared with control (Buffer pH 9.0)

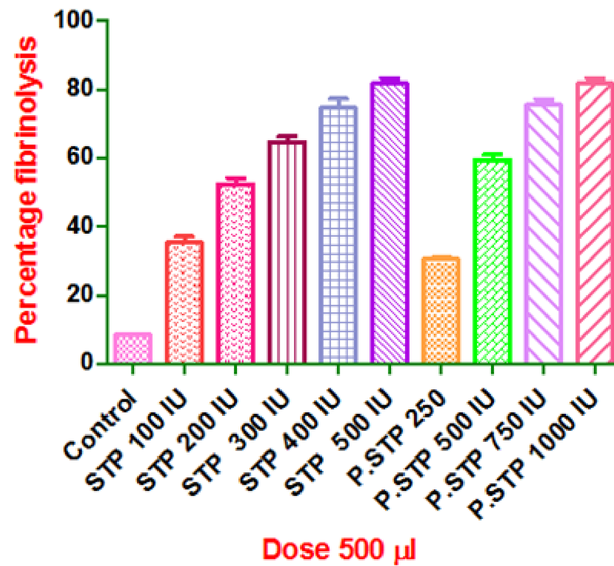

Fig. 4: Percentage fibrinolysis by various concentrations of purified serralysin (P. STP) and commercial serratiopeptidase (STP)
Note: Results are expressed as mean±standard error of mean; n=3 in each group comparison made with control group. Data was analyzed by one way ANOVA followed by Dunnett's test; ***p<0.005 when compared with control (Buffer pH 9.0)

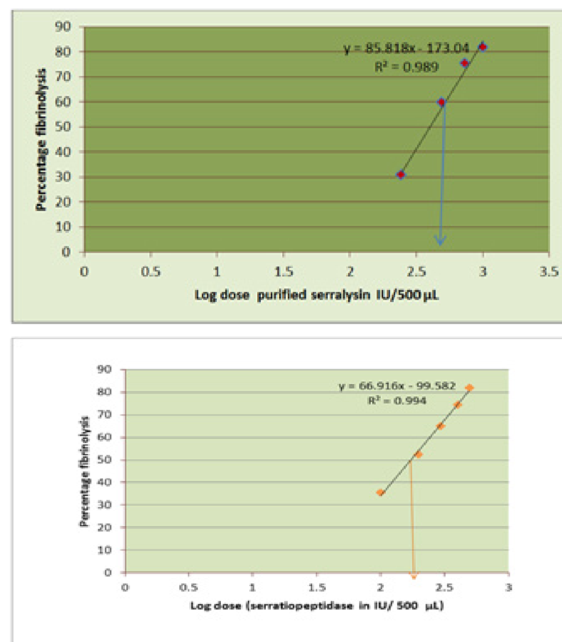

Fig. 5: Log dose (IU/ 500 µl) vs. % fibrinolysis graph of (A): purified serralysin and (B): commercial serratiopeptidase

TABLE 5: PERCENTAGE INHIBITION OF HAEMOLYSIS ON HYPOTONICITY INDUCED HAEMOLYSIS OF HRBC

Concentration (IU/ml)	Purified Serralysin solution	Commercial Serratiopeptidase solution	Diclofenac Solution
100	28.3±4.3	34.4±0.62	-
200	38.0±2.1	44.4±1.1	-
300	42.0±1.3	-	-
400	47.0±1.3	-	-
500	54.3±1.7	-	-
200	-	-	66.6 ± 1.0

Note: Level of significance***p<0.05. Percent inhibition of haemolysis was calculated relative to control. Results are expressed as mean±standard deviation; n=6 in each group comparison made with control group. Data was analysed by one way ANOVA followed by Dunnett's test; ***p<0.05 when compared with control

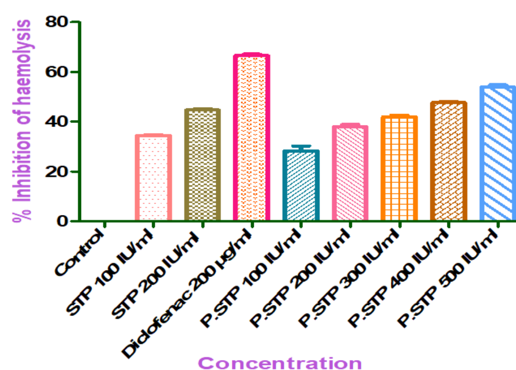

Fig. 6: Percentage inhibition of haemolysis on hypotonicity induced haemolysis of HRBC Commercial seratiopeptidase (STP); Purified serralysin (P. STP). Results are expressed as mean±standard deviation; n=6 in each group comparison made with control group. Data was analyzed by one way ANOVA followed by Dunnett's test; ***p<0.05 when compared with control

The results depicted that the purified serralysin exhibited membrane stabilization effect by protecting the human erythrocyte membrane against hemolysis induced by hypotonic solution. As HRBC membrane is comparable with the lysosomal membrane[37], the percentage inhibition of hemolysis was taken for the assessment of anti-inflammatory activity. Hypotonic solution induces hemolysis by accumulating excess fluid within the cells, this result in rupturing of cell membrane. When there is any damage to RBC membrane, it will make the cell more vulnerable to secondary damage and this damage is occurred due to free radical induced lipid peroxidation. During inflammatory conditions, the vascular permeability of membrane was increased by inflammatory mediators like serotonin, bradykinin and histamine so more fluid is accumulated in tissues. The study revealed that purified serralysin perhaps stabilized the HRBC membrane by hydrolyzing serotonin, bradykinin and histamine. The results are in accordance with other reports where serratiopeptidase obtained from *S. marcescens* protease and L-asparaginase[38-40] obtained from *Aspergillus* sp. also showed excellent membrane stabilizing property.

A cost effective medium was designed for production of serralysin and evaluated for its anti-inflammatory (*in vitro*) and fibrinolytic activity (*in vitro*). The CCD based RSM model was proved to be effective in enhancing serralysin yield by *S. hydrogenans* var. MGS13. The DOE approach assisted in 1.3 fold increases in serralysin yield compared with initial level. The purified enzyme of *S. hydrogenans* var. MGS13 exhibited significant anti-inflammatory and fibrinolytic activity like commercial serratiopeptidase. The biological activities (anti-inflammatory and fibrinolytic) and structural features were similar to serratiopeptidase suggesting it could serves as potential bio-similar for therapeutic and industrial relevance. Further this enzyme formulation may also find many pharmaceutical applications in topical formulation to reduce the inflammation on skin.

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Conflict of interests:

The authors declared no conflict of interests.

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