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Application of Box-Behnken Design for the Optimization of Culture Conditions for Novel Fibrinolytic Enzyme Production by *Bacillus altitudinis* S-CSR 0020

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A bacterium which produced novel extracellular fibrinolytic enzyme for digesting bovine blood clots was isolated from soil, and identified by 16s rRNA sequencing as *Bacillus altitudinis,* given strain name was S-CSR 0020 (accession number KT369312). Fibrin proved the best nitrogen source with an enzyme activity of 750 U/mL, followed by casein after incubation at 37 °C for 4 days. The cultural conditions were optimised using Response Surface Methodology (RSM) and Box-Behnken Design (BBD). Based on 3D surface plot and contour plots, the optimized temperature, pH and substrate concentrations were 47 °C, 10.5 and 4 g/L respectively, resulted in increase in enzyme activity of 306.88 U/mL and specific activity of 780 U/mg which was 2-fold; compared to initial level of 400 U/mg after 2 days of incubation. The crude enzyme has got potent activity and digested human blood clot completely within 1 hr.

Keywords: *Bacillus altitudinis* S-CSR 0020, bovine blood clot, Box Behnken Design, fibrinolytic enzyme.

Fibrin plays a vital role in healing. Inappropriate clotting in blood vessel is a major factor for myocardial infarction and other cardiovascular diseases, resulted in 18 million of people dying every year as reported by World Health Organization. Fibrin, the major component involved in blood clot is formed from fibrinogen by proteolytic activity of thrombin. Meanwhile, plasmin hydrolyzed the clots to avoid thrombosis. But in an unbalanced situation, the fibrin clots are not hydrolyzed, and thrombosis occurs[14]. Twenty enzymes present in the body to assist in clotting of blood, while only the plasmin or any other plasmin like protease can break it down[3]. For the treatment of cardio-vascular diseases, various blood clot-dissolving agents such as urokinase, streptokinase, and tissue plasminogen activator (t-PA) have been utilized[24]. Despite widespread use, these fibrinolytic agents suffer from various side effects including bleeding complications, short half-life, expensiveness, risk of anaphylactic reactions and large therapeutic doses[4], so there is a necessary for the search of new fibrinolytic agents from various sources.

Various proteases from microorganisms have been purified and characterized which are having capability to interfere with the blood clotting mechanism. Microbial fibrinolytic enzymes are classified in to three types: Serine protease,

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Metallo protease and mixture of both Serine and Metallo protease[24]. Fibrinolytic enzyme activity resembles on plasmin, which can degrade fibrin and inhibit clot formation[32]. Many researchers find that high yield of proteases are produced by bacteria[24]. The lack of accurate mathematical model equation to explain the whole process, high noise levels, interaction among variables, and complex biochemical reaction are the obstacles for bioprocess research, needs a good strategy[6] to rectify the problems.

In the past, optimization of media components by one variable at a time, changing one independent variable is a tedious task and it is time consuming, expensive, less accurate to get optimum conditions especially because of interactions among the factors[3, 30]. The limitations of single variable optimization can be eliminated by optimizing the parameters collectively using experimental statistical method such as BBD and RSM[15]. The main objective of this work is production of fibrinolytic enzymes from bacterial soil isolates and to study the effect of various parameters such as temperature, pH and substrate concentration in the yield of fibrinolytic bacterial enzyme by using BBD.

MATERIALS AND METHODS

Chemicals and reagents

All chemicals and reagents of analytical grade were used in this study; purchased from Sigma Aldrich. All experiments were conducted in triplicates and the mean values were considered.

Isolation and cultivation of bacterial isolates

Samples were collected by scraping off the soil from the various locations near slaughter house, Vazhayoor, Malappuram, Kerala, India. Isolation was performed by the soil dilution plate technique[35] on minimal agar medium supplemented with bovine fibrin. Nutrient broth was used to maintain cultures, supplemented with glycerol (70%), and preserved at -20°C[27]. A stock suspension was prepared and adjusted to 7×10^3 cell/ml[10, 33].

Determination of proteolytic activity

The proteolytic activity of the organism was determined by a modified method[11]. Casein agar was prepared by using 1% casein. The organisms were spot inoculated on the medium and incubated at room temperature for 48 hr, the colonies were observed, proteolytic organisms showed a clear hallow around the colony, which indicated the casein hydrolysis by the organism.

Preparation of blood clot for nitrogen source

Buffalo blood was collected from the slaughter house and clot was formed by incubating at room temperature. Then the clots were shredded by using the surgical blade and were placed in 1 L of distilled water in a beaker. The beaker was placed on the magnetic stirrer for further shredding and RBC removal. Water was replaced every half an hour, this procedure was repeated for 2-3 days, until the clot became grey in colour. This clot was grinded into fine powder by using mortar and pestle, sterilized using ethanol and dehydrated by acetone. This powder was used as a source of fibrin and stored at 4 °C in refrigerator.

Screening of fibrinolytic organism

Fibrinolytic organisms were identified by using clot agar with mild modifications[28]. The caseinolytic organisms were spot inoculated on the clot agar medium which contained powdered fibrin. The plates were incubated at 37 °C for 48 hr. Fibrinolytic organisms showed clear zone on fibrin plate agar after adding congo red as an indicator and inoculated into minimal media with 0.5% buffalo fibrin as a substrate, and incubated at 37 °C for 48 hr until the substrate got disappeared, centrifuged and the supernatant was used to check enzyme activity against the substrate fibrin.

Identification of organism

Bacterial colonies were identified macroscopically and microscopically by using staining methods, and by 16s rRNA sequencing, sequence comparison with the databases were performed using BLAST through the NCBI server[29].

Enzyme assay and protein content

Fibrinolytic enzyme activity was estimated[5,8] using 0.5% bovine fibrin in 0.1 M carbonate buffer with an equal volume of extracted enzyme solution and incubated for 30 min at 37 °C. After 30 min, the reaction was stopped by adding 3 mL of 10% cold tri chloroacetic acid (TCA); centrifuged at 5,000 rpm for 10 min. To 0.5 mL supernatant, 2.5 mL of 0.5 M Na_2CO_3 was added, followed by 0.5 mL of two-fold diluted Folin-Ciocalteaure agent; incubated for 30 min at 37 °C and colour developed was read at 660 nm against

a reagent blank. Tyrosine served as the reference standard. The optical density was measured in a UV-Vis (Shimadzu) spectrophotometer. For obtaining the quantitative estimation of the protein content of individual fraction different steps was done systematically[17]. One unit of fibrinolytic activity was defined as the amount of enzyme required to catalyze the release 1 µg of tyrosine per mL under the reaction conditions.

Submerged fermentation using different nitrogen sources

Various nitrogen sources such as casein, fibrin, peptone, yeast extract, skim milk powder were tested for fibrinolytic enzyme production. The enzyme activity and protein content were determined along with the specific activity.

Production and extraction of fibrinolytic enzyme

The organism was inoculated in production media containing minimal media with beef extract (0.3%), casein (1%), buffalo fibrin (0.5%) and peptone (0.5%); pH 7.0, incubated at 37 °C in orbital shaker for 48 hr at 200 rpm. After 76 hr of incubation, the contents of the flasks were filtered through Whatman No.1 filter paper, centrifuged at 5,000 rpm for 10 min and the supernatant was used as the crude enzyme[5].

Statistical optimization of cultural conditions using RSM

A three variable Box-Behnken design is used to study the combined effect of the substrate concentration, broth pH and fermentation temperature on fibrinolytic enzyme production over three levels. The range and levels of the variables optimized are shown in the Table 1. The Box-Behnken design is suitable for the exploration of quadratic response surfaces and generates a second degree polynomial model, which in turn is used in optimizing a process using a small number of experimental runs. This design requires an experimental number of runs according to: $N = k^2 + k + C_p$, where k is the factor number which is 3 in this case and C_p is the number of replications at the center point which is also 3 in this case.

The design which is developed using the Design Expert 10.0.3.1 resulted in 17 experimental runs. The second degree polynomial is expressed as follows:

$Y= b_0 + b_1A + b_2B + b_3C + b_{12}AB + b_{13}AC+ b_{23}BC + b_{11}A^2 + b_{22}B^2 + b_{33}C^2$ (equation 1), where Y is predicted response, A, B and C are independent variables, b_0 is the offset term, b_1, b_2, b_3 are linear effects, and b_{11}, b_{22}, and b_{33} are interaction terms. Regression analysis and analysis of variance (ANOVA) are used to fit the model according to the equation1. Adequacy of models is checked by model analysis and R^2 analysis; F value is checked to find out the significance of all the fitted equation at 5% level of significance. To visualize relationship between response and experimental levels of each factor and to find out optimum conditions, the fitted equations are expressed as contour plots, which is explained by statistical software, Design Expert 10.0.3.1.

Application studies

Blood clot degradation method was used to determine *in-vitro* fibrinolytic activity[19, 7]. Blood was collected from male human volunteer in capillary tube and blood clot was formed by incubating for 1 hr at room temperature. After 1 hr, artificial blood clot in capillary tube was rinsed thoroughly and dipped in 3 mL of carbonate buffer (pH 9.0) containing enzyme solution for clot dissolution, incubated at room temperature and 37 °C, normal saline was kept as control.

RESULTS

Isolation and screening of fibrinolytic organisms

Twenty soil isolates with caseinolytic activity were collected and designated as F1 to F20. Among 20 isolates, 4 isolates exhibited fibrinolytic activity (Fig. 1). Isolate named as F1 with high fibrinolytic activity of 550 U/mL, with a protein content of 0.8 mg and specific activity of 688 U/mg after 4 days of incubation was selected for the further study. Isolates named as F4, F13 and F18 showed less specific activity compared to F1 (Table 2)

On fibrin plate agar after adding congo red, F1 showed clear zone followed by F4 (Fig. 2). Isolate F1 showed clear zone around casein agar (Fig. 3)

Identification of the organism

F1 showed macroscopic appearance of flat, irregular, pale in color, rough (6 to 7 mm) colonies and with a microscopic appearance of large Gram-positive bacilli. From the16S rRNA gene, partial sequence, it was identified as *Bacillus altitudinis* and designated as *Bacillus altitudinis* strain S-CSR 0020, submitted to GenBank

(Accession No. KT369312). The Phylogenetic tree constructed from the sequenced data by the neighbor joining method showed the detailed evolutionary relationship between the strain S-CSR0020 to *Bacillus altitudinis*[29]. The isolate showed 99% similarity with *Bacillus altitudinis.*

Submerged fermentation using different nitrogen sources

Among the nitrogen sources fibrin showed maximum enzyme activity of 750 U/mL with a protein content of 0.69 mg and specific activity of 1087 U/mg of protein followed by casein and peptone by using production medium (Fig. 4 and Table 3).

The best nitrogen sources for the fibrinolytic enzyme production by *Bacillus altitudinis* S-CSR0020 were fibrin and casein.

Statistical optimization of cultural conditions using RSM

Statistical Analysis

Three most important factors such as temperature, pH and substrate concentration are selected for optimization studies. The results obtained from 17 experimental runs carried out according to the Box- Behnken design are summarized in the Table 3. A regression analysis is carried out to fit the mathematical model to the experimental data in order to determine the

Table 1. Actual levels for the factors for three factors Box-Behnken design

Independent Variables	Symbols	Coded and actual levels		
		-1(low)	0	+1(high)
Temperature (^{O}C)	A	10	37.5	65
pH	B	3	6.75	10.5
Substrate Concentration (g/L)	C	0.25	2.125	4

Table 2. Enzyme activity and specific activity of different soil isolates

Soil isolate	Enzyme activity(U/mL)	Specific activity(U/mg)
F1	550	688
F4	450	450
F13	225	205
F18	175	146

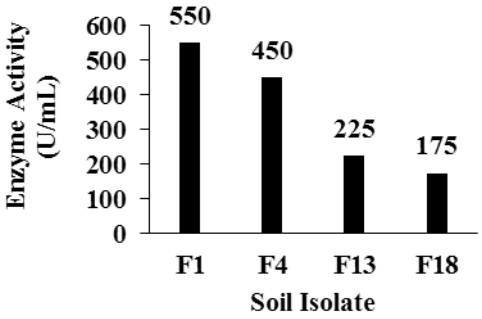


Fig. 1. Enzyme activity of different soil isolates

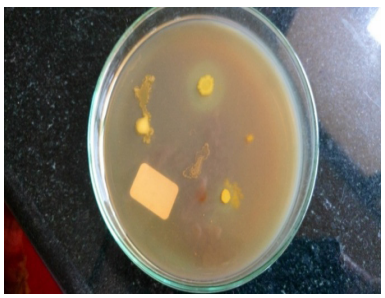


Fig. 2. Fibrinolytic activity of different soil isolates on fibrin plate agar

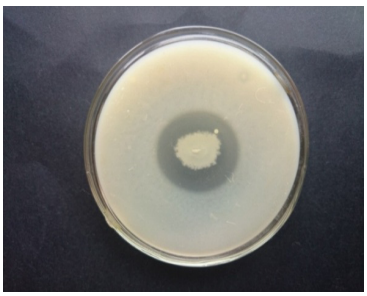


Fig. 3. Caseinolytic activity of F1 on casein agar

optimum fermentation conditions that results in the maximum enzyme production. By applying multiple regression analysis on the experimental data, the following second degree polynomial is found to represent the relationship between enzyme production, substrate concentration, pH and fermentation temperature adequately. The predicted model can be described by the following second-order polynomial equation 1.

Final Equation in Terms of Coded Factors

R1 (U/mL) = + 230 + 45.13 * A + 64.25 * B + 48.63 * C + 25.75 * AB + 28.50 * AC + 20.25 * BC – 102 * A^2 - 27.25 * B^2 -29 *C^2 (equation 1)

Temperature, pH and substrate concentration where represented by A, B and C respectively. The predicted enzyme activity using equation (1) were given in Table 4 with experimental data.

The significance of the fit of the second-order polynomial for enzyme activity is assessed by carrying out analysis of variance (ANOVA) with results are shown in the Table 5.

The coefficient of determination (R^2) of the model is 0.9982 (Table 6), which indicated that the model adequately represented the real relationship between the variables under consideration. The 5ØEÜ^2 value closer to 1.0 shows a stronger model with better predictability. R^2 value of 0.9982 means that 99.82% of the variability was explained by the model and only 0.18 was as a result of chance. The "Pred R-Squared" of 0.9714 is in a reasonable agreement with the "Adj R-Squared" of 0.9959; i.e. the difference is less than 0.2. "Adeq Precision" measures the signal to noise ratio. A ratio greater than 4 is desirable. Ratio of 65.587 indicates an adequate signal. This model can be used to navigate the design space. The coefficient of variation (C.V.) obtained was 3.7.The Coefficient of Variation (C.V.) indicates the degree of precision with which the treatments were carried out. Adequate precision value 65.587 measure the signal to noise ratio and a ratio greater than 4 is desirable.

The Model F-value of 434.06 implies the model is significant. There is only a 0.01% chance that the F-value large could occur due to noise (table 7). Values of "Prob> F" less than 0.05 indicates model terms are significant. In this case A, B, C, AB, AC, BC, A^2, B^2, C^2 are significant model terms, indicating that there is an interaction between temperature and pH, temperature and substrate concentration and pH and substrate concentration.

Optimization of cultural conditions

An increase in temperature and broth pH results in an increase of enzyme activity from about 98.89U/mL to a maximum value of 279.28 U/mL at a temperature of 47.18 °C and broth pH of 10.5 (Fig. 5).

An increase in the substrate concentration and broth pH results in increase of enzyme activity from about 98.89 U/mL to a maximum value of 306.88 U/mL at a substrate concentration of 4 g/L and broth pH of 10.5 (Fig. 6).

An increase in the substrate concentration and temperature results in increase of enzyme activity from about 98.89 U/mL to a maximum value of 262.9 U/mL at a substrate concentration of 4 g/L and temperature of 47.38 °C (Fig. 7)

The response surface methodology is an efficient technique for the rapid screening of the significant influencing parameters and development of a polynomial model to optimize fermentation conditions. The final optimized

Table 3. Enzyme activity and specific activity obtained with different nitrogen source

Nitrogen source	Enzyme activity (U/mL)	Specific activity (U/mg)
Casein	700	1000
Peptone	600	900
Fibrin	750	1087
Yeast extract	470	600
Skim milk powder	300	500

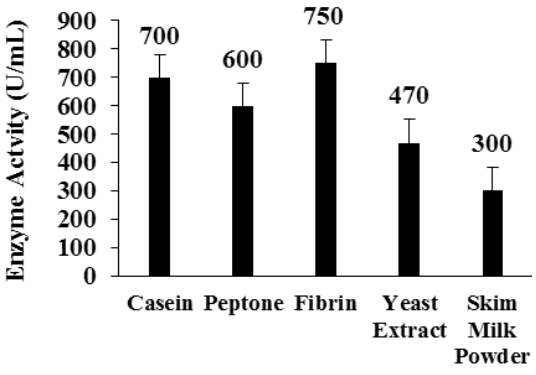


Fig. 4. Effect of different nitrogen sources on enzyme production

fermentation conditions obtain with RSM are 47 °C, 4 g/L (substrate concentration), and 10.5 (broth pH).

Application studies

It shows complete lysis of the blood clot by the crude enzyme happens within 1 hr and from this, it is proved as a good fibrinolytic agent, and activity can be further improved by purification (Fig. 8).

DISCUSSIONS

Fibrinolytic enzyme producing bacilli isolated from soil near slaughter house and the strain showed a clear zone on casein agar and a clear zone on fibrin plate agar after adding congo red indicator. All the fibrinolytic organisms were caseinolytic but not vice versa, so casein could be used for preliminary screening. From the biochemical tests and 16s rRNA sequencing it was designated as *Bacillus altitudinis* S-CSR0020 (accession number KT369312). *Bacillus altitudinis* was first isolated from cryogenic tubes used to collect air samples at altitudes of 41 Km[31]. The isolate obtained in this study was a novel organism since there were no previous reports of the fibrinolytic potential of *Bacillus altitudinis*. Nattokinase could be used as thrombolytic agent, but its high cost and could be used only by intravenous instillation leads to some other alternative method for the large scale production. For fibrinolytic enzyme production, fibrin was a necessary substrate, and it was obtained from blood collected from the slaughter house was an inexpensive substrate for enzyme production. For screening, if the choice for fibrinolytic enzyme production was substrates other than fibrin, then there was a chance of contamination and production of other enzymes. More over fibrin was an insoluble substrate and its utilization by the organism could be detected easily. Enzyme activity preferably could be checked by using fibrin as substrate, because if we used any other assay substrate, there was a chance of misinterpretation of the result. Of the nitrogen sources as growth substrate optimized, fibrin gave maximum enzyme activity of 750 U/mL with a specific activity of 1087 U/mg after incubation for 4 days at 37 °C which was followed by casein. Experiments revealed that incubation time had a significant effect on enzyme production by showing a decrease in enzyme activity of 200 U/mL with a protein content of 0.5 mg and specific activity of 400 U/mg after 2 days of incubation. Marine *Pseudomonas* strain 1452 produced extracellular protease by using casein as carbon as well as nitrogen source[20]. High concentration of nitrogen sources in media was effective in increasing the fibrinolytic enzyme production by *Bacillus cereus* GD55[34]. To date, isolation, productions, characterization of fibrinolytic enzymes were done and there were fewer reports regarding the optimization of media composition especially the major factors like temperature, pH, and substrate concentration. The fermentation temperature was important in that when cells were grown under non ideal temperature, they exhibited

Table 4. Three factor Box – Behnken design with experimental as well as predicted responses of dependent variable

Runs	Actual Values			Enzyme activity (U/ml)	
	A	B	C	Observed	Predicted
1	37.5	6.75	2.125	230.00	230.00
2	37.5	6.75	2.125	230.00	230.00
3	10	10.5	2.125	95.00	94.13
4	65	10.5	2.125	239.00	235.87
5	37.5	6.75	2.125	230.00	230.00
6	37.5	6.75	2.125	230.00	230.00
7	37.5	10.5	4	300.00	306.88
8	37.5	10.5	0.25	172.00	169.13
9	65	6.75	4	225.00	221.25
10	10	6.75	4	80.00	74.00
11	37.5	3	0.25	88.00	81.12
12	10	3	2.125	14.00	17.12
13	10	6.75	0.25	30.00	33.75
14	65	3	2.125	55.00	55.87
15	65	6.75	0.25	61.00	67.00
16	37.5	6.75	37.5	230.00	230.00
17	37.5	3	37.5	135.00	137.88

Table 5. Statistical information for ANOVA

Source	Response Value
R – squared	0.9982
Adjusted R – squared	0.9959
Pred- R square	0.9714
Standard deviation	5.76
C.V %	3.70
Adequate precision	65.587

signs of adverse growth and metabolic production[9]. RSM was a well-known statistical method that employed the cost-effective experimental design and had advantages such as predicted response and evaluation. Statistical experimental design was not very much used in biological sciences rather it was used in chemical, engineering, food science and medical fields[7]. This was designed by using statistical methods to yield the most information in the minimum number of experiments. It had been successfully applied to the optimization of medium composition[7]. Wang et al used BBD to optimize

Table 6. Analysis of variance (ANOVA) for quadratic model of fibrinolytic enzyme production

Sources	Sum of squares	Degrees of freedom	Mean square	F value	P – value [Prob>F]
Model	1.296E+005	9	14401.55	434.06	< 0.0001
A-Temperature	16290.13	1	16290.13	490.98	< 0.0001
B-pH	33024.50	1	33024.50	995.36	< 0.0001
C-Substrate Conc	18915.13	1	18915.13	570.10	< 0.0001
AB	2652.25	1	2652.25	79.94	< 0.0001
AC	3249.00	1	3249.00	97.92	< 0.0001
BC	1640.25	1	1640.25	49.44	0.0002
A^2	43806.32	1	43806.32	1320.32	< 0.0001
B^2	3126.58	1	3126.58	94.23	< 0.0001
C^2	3541.05	1	3541.05	106.73	< 0.0001
Residual	232.25	7	33.18		
Lack of Fit	232.25	3	77.42		
Pure Error	0.000	4	0.000		
Cor Total	1.298E+005	16			

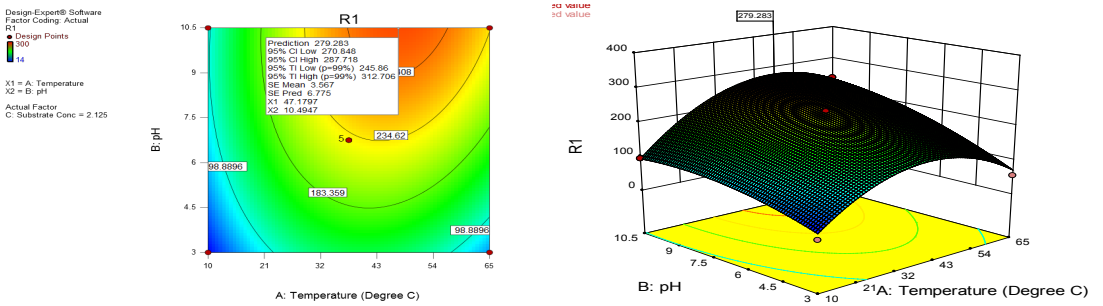


Fig. 5. Contour plot and 3D surface plot showing the effect of temperature and pH on enzyme activity

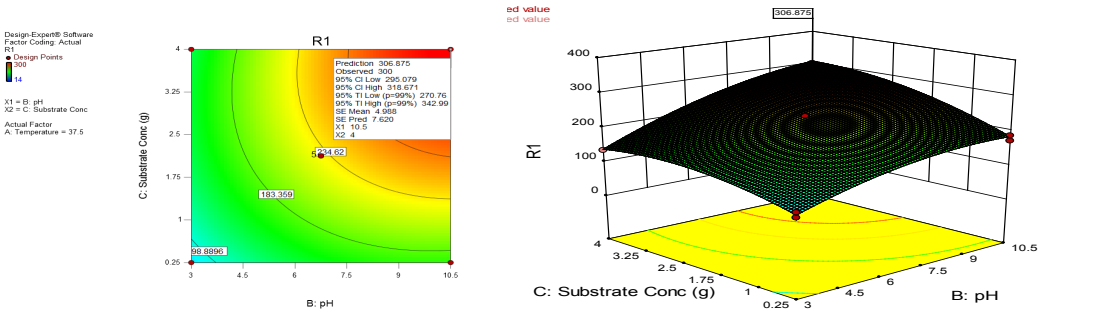


Fig. 6. Contour plot and 3D surface plot showing the effects of substrate concentration and pH on enzyme activity

medium composition for nattokinase production[13]. BBD also used for the optimization of citric acid production by *Aspergillus niger*[1] and dilute acid hydrolysis of cornstover[2]. Fibrinolytic enzyme production from *Bacillus* sp. strain AS-S20-1, *B. natto* NLSSE, and *B. cereus* IND1, were done by the statistical methods such as Plackett–Burman[23], two-level fractional factorial design[16], and two-level full factorial design[25] respectively. RSM could be used to optimize physical environment in terms of temperature and pH and alpha cyclodetrin glucanotranaferase production increased 163.9%[12]. Cost reduction was another factor of physical environment optimization. Regression analysis of the experimental data showed that coefficient of three factors such as temperature (45.13), pH (64.25) and substrate concentration (48.63) were positive (equation 1). Among the three factors pH had the highest impact on fibrinolytic enzyme followed by substrate concentration and temperature. Comparison of experimental and predicted values of the regression model showed the agreement was satisfactory. A low value of C.V. (3.7) suggested a high reliability of the experiment[21, 22]. All the experiments were done in triplicate and the final optimized fermentation conditions obtained by RSM were 47 °C, 4 g/L (substrate concentration), and 10.5 (broth pH), with a predicted maximum enzyme activity of 306.88 U/mL after an incubation of 2 days. Further increase of temperature, pH, and substrate concentration had a reverse effect on enzyme production. The results obtained from three replications showed that the average of experimental value, 312 U/mL is close to mathematically predicted value, suggested that the model was valid. The protein content was noticed as 0.4 U/mg with a specific activity of 780 U/mg after 2-day incubation suggested that optimization of cultural conditions by statistical approach gave an overall 2 fold increase in enzyme

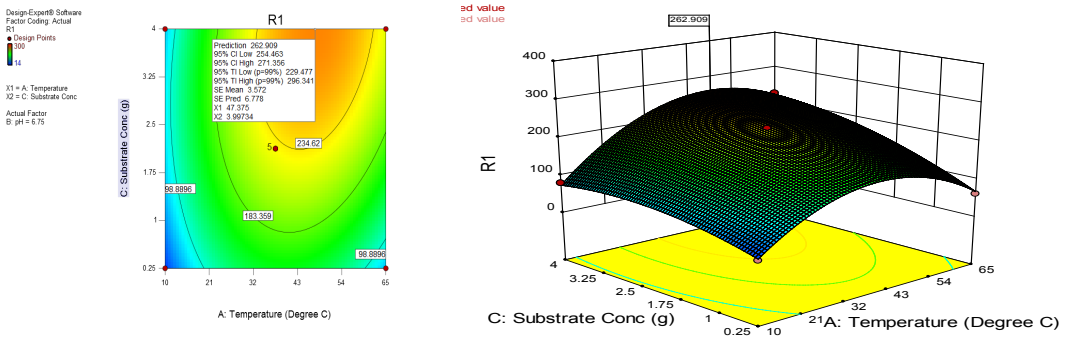
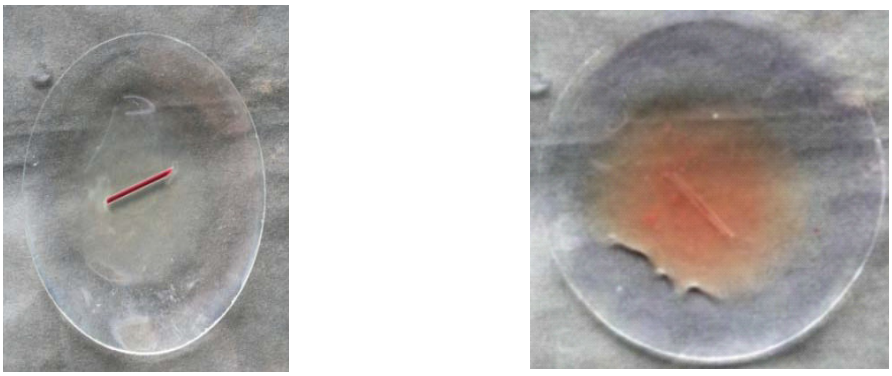


Fig. 7. Contour plot and 3D surface plot showing the effects of fermentation temperature substrate concentration on enzyme activity



Control (blood clot with normal saline) Test (blood clot with enzyme solution)

Fig. 8. Watch glass showing blood clot lysis

production. Nattokinase production dependent on magnesium sulfate, dipotassium hydrogen phosphate, and yeast extract by using BBD[13]. From the results, it was found that the organism is temperature and pH stable and it could produce enzyme at extreme conditions. Further factors like carbon source, minerals, and incubation period had to be optimized and purification steps had to be done to increase the enzyme activity further. The novel fibrinolytic enzyme of *Bacillus altitudinis* S-CSR0020 showed high blood clot lytic activity, a clot lysed completely within 1 hr by the crude enzyme. Purified enzyme from an *Actinomycete* showed lysis of clot weighing 0.41 g within two hour[28]. *In -vitro* blood clot lysis studies were performed with other organisms after purification to an extent[6, 18, 16, 26, 36]. By purification, this enzyme could be used as a promising fibrinolytic agent to treat myocardial infarction and other cardiovascular diseases in future.

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