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Response Surface Methodology Based Optimization of a New Isolate *Bacillus pumilus* ZR LS S2 with Fibrinolytic Activity

C. V. Reshma[1*] and K. Fathimathu Zuhara[1]

[1]Department of Life Sciences, University of Calicut, Malappuram, Kerala, India.

Authors' contributions

This work was carried out in collaboration between both authors. Both authors read and approved the final manuscript.

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ABSTRACT

Aims: The work aims to isolate potential fibrinolytic enzyme producing isolates from various samples with the aim of developing a suitable optimization strategy using response surface methodology.
Study Design: Plackett- Burman and Face centered central composite design was used.
Place and Duration of Study: Department of Life Sciences, University of Calicut.
Methodology: Various samples were screened using fibrin agar plates for the isolation of fibrinolytic enzyme producing bacteria and was identified by 16 s rRNA sequencing. Further the physical parameters and media components were optimized using Plackett- Burman design and face centered central composite design.
Results: A novel isolate with fibrinolytic potential was isolated and was identified as *Bacillus pumilus* ZR LS S2. A novel media was formulated using this data, for the production of fibrinolytic enzyme, which contains peptone, casein, $MgSO_4$ and NaCl. The isolate produced 6738.384 U/ml of enzyme in optimized conditions.
Conclusion: Initial studies were performed for the production of the fibrinolytic enzyme by the novel isolate, further studies are required to effectively validate the potential of the fibrinolytic enzyme and to develop effective application for the same.

**Corresponding author: Email: reshmavalsalan@gmail.com;*

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1. INTRODUCTION

With the evolution of vertebrates and their pressurized circulatory system, there had to arise some method to seal the system if injured—hence the hemostatic system [1]. The normal state of circulatory system is that of a conduit through which blood flows in a liquid phase until vascular injury occurs and at which time haemostatic mechanisms operate to prevent the defect [2]. Thrombosis is the pathological condition in which haemostasis is excessively activated in the absence of bleeding which results in the formation of abnormal blood clots [3].

The key element in clot formation is the protein fibrin, which forms the network in a clot in which other elements are enmeshed. Fibrin is produced from fibrinogen, which are 45 nm elongated structures with two sets of three polypeptide chains; Aα, Bβ and γ which are joined together in the N terminal E domain by five symmetrical disulphide bridges. Cleavage by thrombin of a small fibrinopeptide from the N terminal sequence of Aα chain leads to the initiation of fibrin assembly process [4].

The formation and disruption of the fibrin network is balanced in a healthy body. The dissolution of blood clot is aided by the serine protease plasmin which is formed from plasminogen a plasma zymogen. However in some cases the balance is disrupted and the formation of the blood clot often leads to the development of disease conditions in the system, Cardiovascular diseases (CVDs). According to recent estimates, the cases of CVDs may increase from 2.9 crore in 2000 to as many as 15 crore in 2015. Cardiovascular diseases are major causes of mortality and disease in the Indian subcontinent, causing more than 25% of deaths. It has been predicted that these diseases will increase rapidly in India and this country will be host to more than half the cases of heart disease in the world within the next 15 years [5].

Fibrinolytic enzymes have a significant role in treating the CVDs. Based on their mechanism of action fibrinolytic enzymes are classified in to two, plasmin like proteins and plasminogen activators. Plasminogen activators activates plasminogen to active plasmin which degrade fibrin. Tissue type plasminogen activators and urokinase are examples of this type of fibrinolytic agents.The second type of agents acts like plasmin which directly degrade fibrin thereby digesting the clot completely and rapidly [6].

Fibrinolytic enzymes have been isolated and characterized from different sources like lumbrokinase from earth worms, fibrolase from snake venom, plants, marine microorganisms, invertebrates etc [7-9]. One of the suitable sources of fibrinolytic enzyme are microorganisms [10]. The most rewarding starting point for the search and discovery of the product is the rich diversity of microorganisms in nature. It is seldom appreciated that for 85% of the Earth's history, life was restricted to microbial forms. The metabolic diversity this has created is truly immense [11]. Microbial enzymes are preferred because they are generally cheaper to produce, their enzyme content is more predictable and controllable and reliable supplies of raw materials of constant composition are available as raw materials for enzyme production. The agents that are isolated so far possess significant shortcomings like low specificity, hypersensitivity, high cost etc., so that the search for a potential fibrinolytic agent continues throughout the world to alleviate the burden of cardiovascular diseases.

Once the initial cultivation of the desired microbe is achieved, the medium is frequently subjected to optimization. The process of optimization usually refers to finding the conditions, appropriate nutrients, nutrient concentration, aeration etc that will support the best cell growth or synthesis of a microbial product.

Two approaches are practiced for medium optimization, one is the change of one variable at a time, classical method; and the other one is the change of one or more variables from one test to the next; statistical approach. One at a time approach was simple and easy but it fails to locate the region of optimum response because in such procedures the joint effects of factors on the response are not considered. The complexities and uncertainties associated with large-scale fermentation usually come from lack of knowledge of the sophisticated interactions among various factors [12].

The aim of this study was the isolation of a potential fibrinolytic enzyme producer and optimization of fibrinolytic enzyme production through statistical approach.

2. MATERIALS AND METHODS

2.1 Chemicals

All the chemicals and culture media used in the present study were of the AR grade. Fibrinogen and thrombin were purchased from Sigma Aldrich; USA. Buffer salts like Tris, NH_4NO_3, $MgSO_4$, KH_2PO_4, NaCl, $FeSO_4$, soya peptone, HCl etc. were purchased from Himedia India Limited, Mumbai.

2.2 Isolation and Screeining of Fibrinolytic Enzyme Producing Bacteria

Different samples were collected like soil samples, food items, vegetables, butchery waste etc with the aim of isolating fibrinolytic enzyme producer. The samples were homogenized in saline and spread on to casein agar media for detection of protease production. For screening of fibrinolytic enzyme producers, modified method of Astrup and Mullertz was followed [13]. The protocol used fibrinogen (0.5%) in 50mM Tris HCl buffer (2 ml) with 1 µl of thrombin and was mixed with sterile molten agar (2 ml of 1% agar in distilled water) and allowed to soldify in a petridish, positive protease producers were spot inoculated onto fibrin agar plates and incubated at 37°C to determine the hallow zone which is indicative of fibrin digestion. From this, the colony with highest zone of clearance was selected for further studies.

2.3 Biochemical Analysis and 16s rRNA Sequencing of Fibrinolytic Enzyme Producing Strain

2.3.1 DNA isolation and PCR

The DNA sample was isolated using the Sambrook et al., method [14]. The desiccated DNA samples were completely resuspended in 50 µl of DNA dissolving buffer (TE buffer) and stored at -80°C.

PCR was carried out in a final reaction volume of 25 µl in 200 µl capacity thin walled PCR tube. Composition of reaction mixture was Deionized water - 16.5 µl, Taq buffer without $MgCl_2$ (10 X)- 2.5 µl, $MgCl_2$ (15 mM) -1.0 µl, dNTPs mix (10 mM each) - 1.5 µl. PCR tubes containing the mixture were tapped gently and spinned briefly at 10,000 rpm. The PCR tubes with all the components were transferred to thermal cycler. The concentration of the purified DNA was determined and was subjected to automated DNA sequencing on ABI3730xl Genetic Analyzer (Applied Bio systems, USA).

2.4 Response Surface Methodology Based Optimization of Physical Factors and Media Components

Optimization of physical parameters was carried out using nutrient broth supplemented with casein as the production medium. The process parameters pH, temperature and agitation were optimized statistically using the full factorial Face Centered Central Composite Design (FCCCD) of the RSM. The software Minitab 14 was used for experimental design, data analysis and the quadratic model building. The optimal levels of the variables were obtained by solving the regression equation and by analyzing the response surface contour plots using the same software.

For the optimization of media components initially Plackett- Burman model was used to determine the significant variables, the variables were peptone, soy peptone, yeast extract, casein, $MgSO_4$, $(NH_4)_2NO_3$, KH_2PO_4, $FeSO_4$ and NaCl (Table 1).

Table 1. Levels of the variables tested in Plackett- Burman design

Variables g/L	Symbol	Coded levels	
		Low	High
Peptone	A	0	5
Soy peptone	B	0	10
Yeast extract	C	0	3
Casein	D	.1	10
$MgSO_4$	E	0.005	0.5
NH_4NO_3	F	0.03	0.3
KH_2PO_4	G	0.002	0.2
NaCl	H	0.5	5
$FeSO_4$	I	0.001	0.1

Based on the results of Plackett- Burman model, the variables were selected for FCCCD and they were casein, peptone, $MgSO_4$ and NaC. All the variables were assessed at three coded levels, +1, -1 and 0. Thirty one experimental runs were carried out with six replicates at the centre points (Table 2). Media were prepared in all the combinations in conical flasks, inoculated and incubated at the specific temperature and RPM as specified by the model. The fibrinolytic enzyme assays were carried out by slightly modified method of Wu, to determine the enzyme

levels. 1.3 ml of Tris–HCl (50 mM, pH 7.5) and 0.4 ml of 0.72% (w/v) fibrinogen solution were taken in vials and kept in water bath (37°C) for 5 min. Then 0.1 ml thrombin (20 U/ml) was added and incubated in water bath (37°C) for 10 min to develop fibrin clots. To this clot, 0.1 ml of enzyme was added. After incubation (37°C, 60 min), 2 ml of 0.2 M trichloroacetic acid (TCA) was added. Vials were kept 20 min. Absorbance at 280 nm for the supernatant was measured and converted to the amount of tyrosine equivalent. One unit enzyme activity is defined as the amount enzyme releasing 1 µmol of tyrosine equivalent per hour [15].

The statistical significance of the model equation and the model terms were evaluated via Fisher's test. The quality of fit of the second-order polynomial model equation was expressed via the coefficient of determination, R^2 and the adjusted R^2. The fitted polynomial equation was then expressed as three-dimensional surface plots to illustrate the association between the responses and the tentative levels of each of the variables utilized in this study.

3. RESULTS AND DISCUSSION

3.1 Isolation and Identifiction of the Fibrinolytic Enzyme Producer

The samples collected from various sources were diluted in saline and spread plated on to casein agar plates to determine the protease production. Around 72 colonies were obtained and were further screened for fibrinolytic enzyme production on fibrin agar plates. Of the 72 colonies, 6 showed fibrinolytic enzyme production and the isolate showing the highest zone of clearance on fibrin agar was selected for further optimization studies.

For the identification, polyphasic approach was performed including morphological, physiological, biochemical tests (data not shown) and 16 s rRNA sequencing. The results indicated that the isolate was *Bacillus pumilus* ZR LS S2. The sequence was deposited in NCBI (Accession number: KJ509594.1).

3.2 Response Surface Methodology Based Optimization of Physical Parameters and Media Components

The enzyme production varies according to different physical parameters for the isolate. The actual value of the enzyme is close to that predicted by the software. The experiments were done in triplicates and the actual values were the averages of these experiments. The results obtained after FCCD (Table 3) were subjected to ANOVA (Tables 4, 5) which gave the regression equation for the enzyme production as a function of the variables that is temperature, pH and agitation speed.

Table 2. FCCCD matrix for media components for *Bacillus pumilus* ZR LS S2

Run order	Coded levels							
	Casein		Peptone		$MgSO_4$		NaCl	
1	0	5.05	0	5.05	+1	0.5000	0	2.525
2	+1	10.00	-1	0.10	-1	0.0050	-1	0.05
3	0	5.05	0	5.05	-1	0.0050	0	2.525
4	-1	0.10	-1	0.10	-1	0.0050	+1	5.000
5	0	5.05	+1	10.00	0	0.2525	0	2.525
6	-1	0.10	+1	10.00	+1	0.5000	-1	0.050
7	-1	0.10	-1	0.10	-1	0.0050	-1	0.050
8	-1	0.10	-1	0.10	+1	0.5000	+1	5.000
9	-1	0.10	+1	10.00	-1	0.0050	+1	5.000
10	+1	10.00	-1	0.10	+1	0.5000	+1	5.000
11	+1	10.00	-1	0.10	+1	0.5000	-1	0.050
12	0	5.05	0	5.05	0	0.2525	0	2.525
13	0	5.05	0	5.05	0	0.2525	-1	0.050
14	0	5.05	0	5.05	0	0.2525	+1	5.000
15	0	5.05	0	5.05	0	0.2525	0	2.525
16	+1	10.00	+1	10.00	+1	0.500	-1	0.050
17	0	5.05	0	5.05	0	0.2525	0	2.525
18	0	5.05	0	5.05	0	0.2525	0	2.525
19	-1	0.10	0	5.05	0	0.2525	0	2.525
20	0	5.05	0	5.05	0	0.2525	0	2.525
21	0	5.05	-1	0.10	0	0.2525	0	2.525
22	+1	10.00	+1	10.00	-1	0.0050	+1	5.000
23	0	5.05	0	5.05	0	0.2525	0	2.525
24	+1	10.00	-1	0.10	-1	0.0050	+1	5.000
25	-1	0.10	+1	10.00	+1	0.5000	+1	5.000
26	+1	10.00	0	5.05	0	0.2525	0	2.525
27	0	5.05	0	5.05	0	0.2525	0	2.525
28	-1	0.10	+1	10.00	-1	0.0050	-1	0.050
29	-1	0.10	-1	0.10	+1	0.5000	-1	0.050
30	+1	10.00	+1	10.00	+1	0.5000	+1	5.000
31	+1	10.00	+1	10.00	-1	0.0050	-1	0.050

Enzyme activity(Y): -4287.25+ -1.88 A+ 540.69 * B+146.42 * C*$A^2$0.01* B^2 -28.33* C^2 -2.10A * B -0.02* A * C 0.01*BC-0.05*
(Regression equation for physical factors)

Table 3. Face centered central composite design for physical parameters: *Bacillus pumilus*

Run order	Agitation (RPM)	pH	Temperature (°C)	Enzyme activity (U/ml)	Predicted value
1	110	9.5	25	530.45	537.45
2	200	5	45	63.7	64.748
3	110	9.5	35	745.32	746.118
4	200	9.5	35	813.3	814.104
5	110	14	35	150.2	151.272
6	110	9.5	45	521.8	522.862
7	110	9.5	35	745.2	746.118
8	200	14	45	1.9	2.585
9	110	9.5	35	745.8	746.118
10	110	9.5	35	746.3	746.118
11	200	5	25	5.95	6.941
12	20	14	25	32.87	33.752
13	110	5	35	176.2	178.12
14	20	14	45	12.93	53.231
15	110	9.5	35	746.9	746.118
16	200	14	25	1.9	7.387
17	20	9.5	35	794.2	795.368
18	20	5	45	0.678	13.863
19	110	9.5	35	745.32	746.118
20	20	5	25	24.98	25.285

Table 4. Estimated regression coefficients for *Bacillus pumilus* ZR LS S2

Term	Coefficient	SE coefficient	T value	P value
Constant	-4287.25	105.584	-40.605	*0.000*
Agitation (A)	-1.88	0.343	-5.502	*0.000*
P^H (B)	540.69	9.453	57.195	*0.000*
Temperature (°C)	146.42	6.379	22.955	*0.000*
A*A	0.01	0.001	7.455	*0.000*
B*B	-28.33	0.440	-64.429	*0.000*
C*C	-2.10	0.089	-23.552	*0.000*
A*B	-0.02	0.013	-1.218	*0.251*
A*C	0.01	0.006	1.155	*0.275*
B*C	-0.05	0.116	-0.393	*0.703*

Table 5. ANOVA for physical parameters

Source	DF	SEQ SS	Adj SS	Adj MS	F	P
Regression	9	2297997	2297997	255333	1171.18	*0.000*
Linear	3	166	1086289	362096	1660.88	*0.000*
Square	3	2297183	2297183	765728	3512.28	*0.000*
Nteraction	3	648	648	216	0.99	*0.436*
Residual error	10	2180	2180	218	-	*0.000*
Lack of fit	5	1392	1392	278	1.77	*0.274*

Based on the Plackett- Burman design casein, peptone, $MgSO_4$ and NaCl were found to be the factors significantly affecting the enzyme production for *Bacillus pumilus* ZR LS S2. ANOVA was used to find out the effects of media components on production of fibrinolytic protease (Tables 6, 7). The values for *Bacillus pumilus* suggested that the regression model was significant with high F value and a low probability value. 0.994 is the R^2 value and it is suggested high goodness of fit of the model and only 0.6% of the total variations are not explained by the model and the adjusted R^2 is also high(0.99). The lack of fit value was also high and all these

factors indicated that the regression is highly significant.

4. DISCUSSION

Microorganisms known to produce an array of enzymes and many of them are useful in a variety of ways to human life. The scientific world is behind such life forms to explore the unidentified potential of microorganisms. Fibrinolytic enzyme producers were isolated by many workers and the quest for these enzymes has not been stopped. In our study also the different sources were screened with the perspective of isolating a potent fibrinolytic enzyme producer from the microscopic life.

From the sources selected, fibrinolytic enzyme producing organisms were selected based on their zone of clearance on fibrin agar plates. The isolate that showed a clear zone of 2.9 cm was further selected for the detailed study, since agar diffusion tests provide qualitative and very rough quantitative estimate of the enzyme production capacity.

The biochemical, physiological, morphological and 16 s r RNA sequencing studies identified the isolate as *Bacillus pumilus* ZR LS S2 (Fig. 1). *Bacillus sp* are producers of important extracellular enzymes, including proteases. Their capacity to produce gram quantities of enzymes per litre of the culture medium and high growth rate makes these organisms an excellent source for enzyme production. *Bacillus* species are good secretors of proteins and metabolites [16,17].

There are reports showing various species of *Bacillus* producing extracellular fibrinolytic enzymes. A novel fibrinolytic enzyme was purified from *Bacillus* subtilis natto *B12* by Wang [18]. Mahajan et al. isolated a *Bacillus* subtilis strain from marine environments while Jo et al., isolated a fibrinolytic enzyme producing *Bacillus amyloliquefaciens* from meju [19,20].

The increasing demand for fibrinolytic enzymes necessitated the conducting of optimization studies very seriously, for the maximization of the enzyme yield. The objective of the present study was to investigate the effect of different physical parameters and media components on fibrinolytic enzyme production of the newly isolated organisms thereby selecting the conditions and media for the optimum production of fibrinolytic enzymes. On a large industrial scale the production of secondary metabolites by microorganisms are practiced in submerged fermentations because of the fact that this system has several advantages such as proper agitation and mixing of substrate, easy to control moisture level, dissolved oxygen, pH level, aeration level, proper monitoring of microbial growth [21]. In view of these, this study also followed submerged fermentation for optimization studies.

Statistical means were used in the process instead of classical approaches. Only very few studies are carried out in this area using statistical tools. Statistical experimental design has not been widely used in the biological sciences even though it has been unanimously employed in numerous other areas such as business, chemical, industrial, agricultural, medical, and food sciences [22].

Table 6. Plackett–burman design matrix for media components along with predicted and obtained values

Run order	A	B	C	D	E	F	G	H	I	Enzyme activity(U/ml)	Predicted value
1	-1	1	1	1	-1	1	1	-1	1	473.89	475.66
2	-1	1	1	-1	1	-1	-1	-1	1	275.29	277.43
3	-1	-1	-1	1	1	1	-1	1	1	219.75	220.75
4	-1	-1	1	1	1	-1	1	1	-1	480.45	481.27
5	1	-1	-1	-1	1	1	1	-1	1	564.98	650.68
6	1	-1	1	1	-1	-1	1	1	1	1289.76	1589.86
7	1	1	-1	1	-1	-1	-1	1	1	3452.96	4959.16
8	1	1	-1	1	1	-1	1	-1	-1	73.18	75.18
9	1	-1	1	-1	-1	1	-1	-1	-1	1564.98	1578.85
10	-1	-1	-1	-1	-1	-1	-1	-1	-1	578.89	588.37
11	1	1	1	-1	1	1	-1	1	-1	1298.45	1446.87
12	-1	1	-1	-1	1	1	1	1	-1	34.98	-418.78

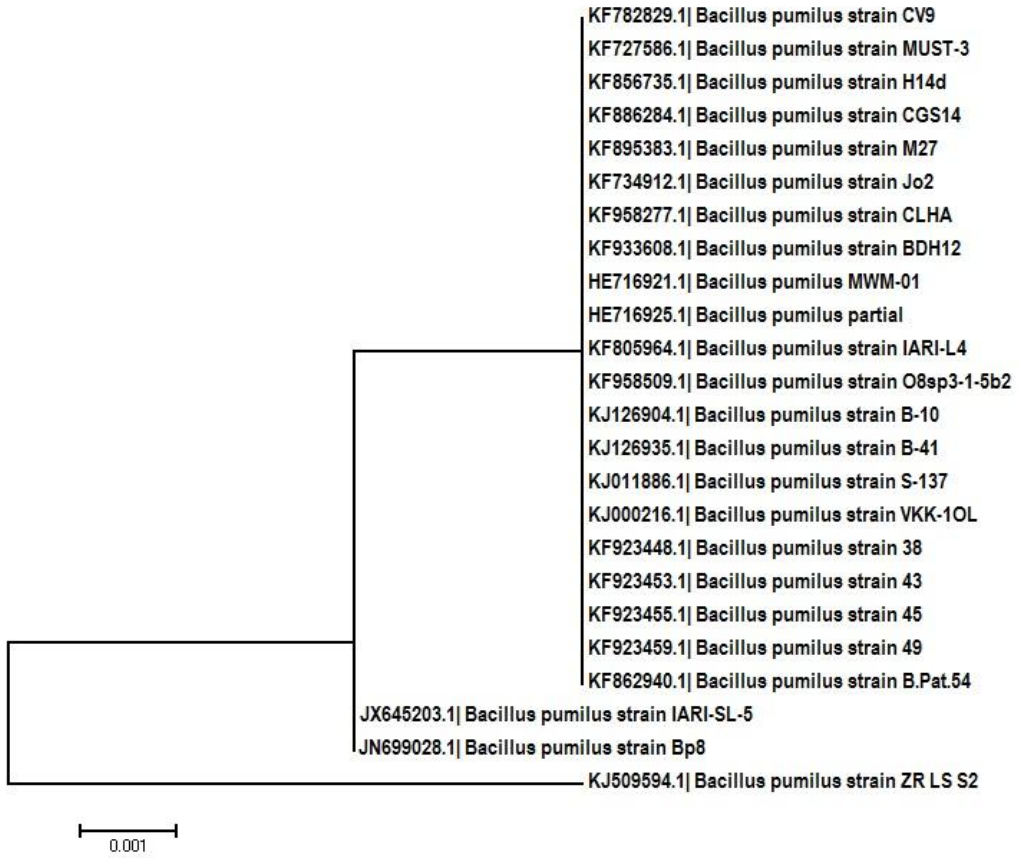

Fig. 1. Phylogenetic relationship of isolated strain ZR LS S2 with other *Bacillus pumilus* strains

Table 7. ANOVA for media components

Term	Effect	Coef	SE coef	T	P
Constant		993.8	27.28	36.43	0.001
Peptone	954.8	477.4	27.28	17.50	0.003
Soy peptone	540.9	270.5	27.28	9.92	0.010
Yeast extract	-967.9	-483.9	27.28	-17.74	0.003
Casein	815.0	407.5	27.28	14.94	0.004
$MgSO_4$	834.5	417.3	27.28	15.30	0.004
$NH_4 NO_3$	-869.9	-434.9	27.28	-15.95	0.004
NaCl	830.9	415.5	27.28	15.23	0.004
KH_2PO_4	-668.4	-334.2	27.28	-12.25	0.007
$FeSO_4$	-779.1	-389.6	27.28	-14.28	0.005

S = 94.4891 R-Sq = 99.90% R-Sq adj = 99.46%

In this study physical parameters and media components are found to influence the fibrinolytic enzyme production and the conditions are optimized for the enzyme production. The effect of process parameters like temperature, pH and agitation were selected for their effect on fibrinolytic enzyme production by the two isolates and it was found that these parameters have an impact on the activity. The quadratic regression model for *Bacillus pumilus* ZR LS S2 suggested that it is highly significant as it had an F value of 1171.18 and a probability of 0.000 which indicates that the model is highly significant (Table 5). The goodness of fit of the model was determined from the value of R^2 (multiple correlation coefficient/determination coefficient), which gives measure of how much variability in the observed response could be explained by the environmental/physical factors and their interactions. This value was very high for this model (0.998) which indicates that 99.8% variation can be explained, while only 0.2% cannot be explained by the model. R^2 values more than 0.9 indicates a high correlation and a model value with R^2 value between 0.7 and 0.9 is considered as having a good correlation [23,24]. So in this case an R^2 value of 99.8% reflected a good fit between the observed and predicted responses and it was reasonable to use the regression model to analyze the trends in the responses.

From the data generated, the solution was suggested by the system for optimum production of enzyme for *Bacillus pumilus* ZR LS S2, an agitation speed of 170, pH of 7.5 and a temperature of 35°C was found to be optimum. These predicted conditions will produce an enzyme activity of 686.0119 and the obtained activity for the same is 674.48 U/ml.

Based on the results of the Plackett- Burman matrix, the variables selected for the optimization of media and are casein, peptone, magnesium sulphate and sodium chloride, which are further optimized by FCCCD.

ANOVA results of *Bacillus pumilus* ZR LS S2 suggested that the media components casein, peptone, $MgSO_4$ and NaCl had profound effect on the enzyme production. The quadratic terms i.e. A^2 (Casein), B^2 (Peptone), C^2 ($MgSO_4$) & D^2 (NaCl) were also effective and the coefficients of the three terms A^2 (Casein), B^2 (Peptone) & D^2 (NaCl) were found to be negative, suggesting that the elevated concentrations of the above factors negatively influence enzyme production (Tables 8, 9). Contour and response surface graphs were plotted from the data obtained after the ANOVA and regression analysis (Figs. 2, 3).

The repression of enzyme production by the presence of high concentrations of nitrogen sources have been reported by many workers. It was proposed that the extracellular protease enzymes from *Bacillus* are a sign of nitrogen restriction at the commencement of stationary phase [25]. Complex organic nitrogen sources similar to yeast extract and peptone that is rich in amino acids and small peptides displayed enzyme repression when they are used in high concentrations [26]. This may be the reason for the negative values for the casein and peptone.

Wang et al., observed that magnesium sulphate is the inorganic metal ion, which shows positive effect on fibrinolytic enzyme production [27]. In our study also the same compound was necessary for optimum production of enzyme.

The RSM gave the following regression equation for the fibrinolytic enzyme production Y, as a function of casein (A), Peptone (B), $MgSO_4$ (C) and NaCl (D).

Enzyme activity Y = +956.171 +1516.08 * A + 940.047 * B - 18536.6 *C -54.4030*D * A^2 - 106.242 * B^2 -93.0149+ 33565.6 * C^2 -39.6455D^2 * A * B-26.2949 * A * C - -385.554 * B * C - 90.4520+ 18.7042*B*+C481.532*C*D

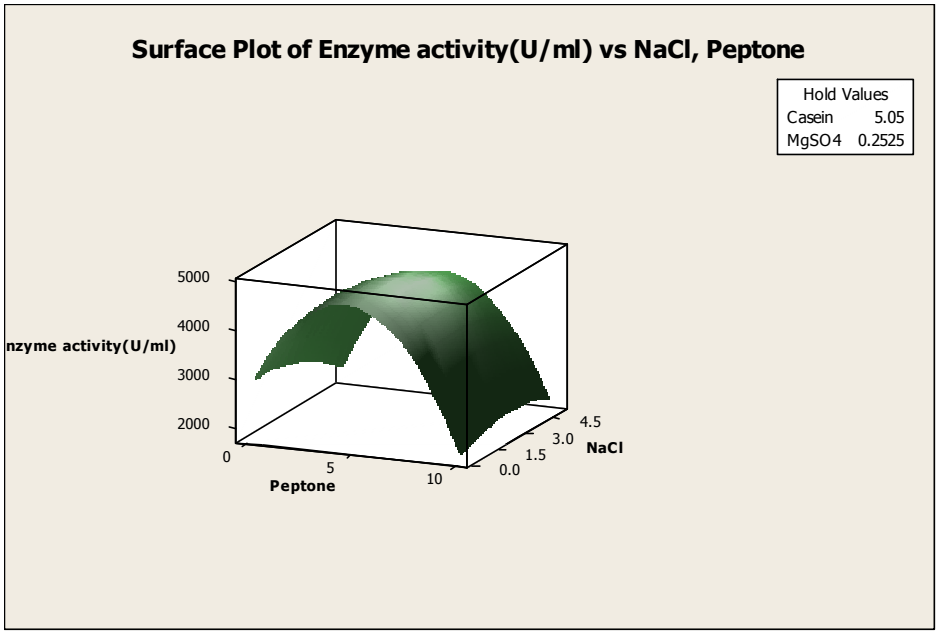

Fig. 2. Surface plot of enzyme activity vs NaCl and peptone

Table 8. FCCD results for *Bacillus pumilus* ZR LS S2 with actual and predicted values for fibrinolytic enzyme activity

Run order	Casein	Peptone	$MgSO_4$	NaCl	Enzyme activity (U/ml)	
					Predicted value	Actual value
1	0	0	+1	0	6355.04	6569.9
2	+1	-1	-1	-1	5442.49	5370.98
3	0	0	-1	0	7275.9	6985.47
4	-1	-1	-1	+1	-137.63	5.87
5	0	+1	0	0	2172.75	2010.45
6	-1	+1	+1	-1	754.03	906.98
7	-1	-1	-1	-1	1104.9	1064.45
8	-1	-1	+1	+1	254.52	312.96
9	-1	+1	-1	+1	772.58	723.92
10	+1	-1	+1	+1	2369.33	2127.45
11	+1	-1	+1	-1	2765.36	3018.39
12	0	0	0	0	4759.37	4818.93
13	0	0	0	-1	4697	4768.92
14	0	0	0	+1	4336.02	4188.54
15	0	0	0	0	4759.37	4818.51
16	+1	+1	+1	-1	625.07	296.09
17	0	0	0	0	4759.37	4818.51
18	0	0	0	0	4759.37	4808.19
19	-1	0	0	0	1187.36	1218.43
20	0	0	0	0	4759.37	4789.23
21	0	-1	0	0	2787.79	2874.52
22	+1	+1	-1	+1	2199.63	2253.43
23	0	0	0	0	4759.37	4801.89
24	+1	-1	-1	+1	3866.58	3918.01
25	-1	+1	+1	+1	1607.98	1494.02
26	+1	0	0	0	3124.98	3018.34
27	0	0	0	0	4759.37	4687.02
28	-1	+1	-1	-1	1098.5	1154.9
29	-1	-1	+1	-1	317.17	77.89
30	+1	+1	+1	+1	1145.63	1390.45
31	+1	+1	-1	-1	2858.94	3004.87

Table 9. Analysis of variance (ANOVA) for the fitted quadratic polynomial model of fibrinolytic protease production by *Bacillus pumilus* ZR LS S2

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Regression	14	120526327	120526327	8609023	204.99	0.000
Linear	4	22999286	22999286	5749822	136.91	0.000
Square	4	84775551	84775551	21193888	504.64	0.000
Interaction	6	12751490	12751490	2125248	50.60	0.000
Residual error	10	16	671964	671964	41998	-
Lack-of-Fit	10	658445	658445	65845	29.22	0.000
Pure error	30	6	13519	13519	2253	-
Total	30	121198291				-

DF – Degrees of freedom, S = 204.9 R-Sq = 99.4% R-Sq (adj) = 99.0%

The validation of the model was done, The value obtained was 6368.398 U/ml and the predicted value was 6379.9210 u/ml (Table 10), the data obtained was very near to the predicted value suggesting that the model was good.

This part of work is of high relevance because if isolates are to be used for industrial purposes the media for the production of enzymes should be optimized so that the wastage of essential nutrients could be avoided which otherwise would lead to economic loss in the process.

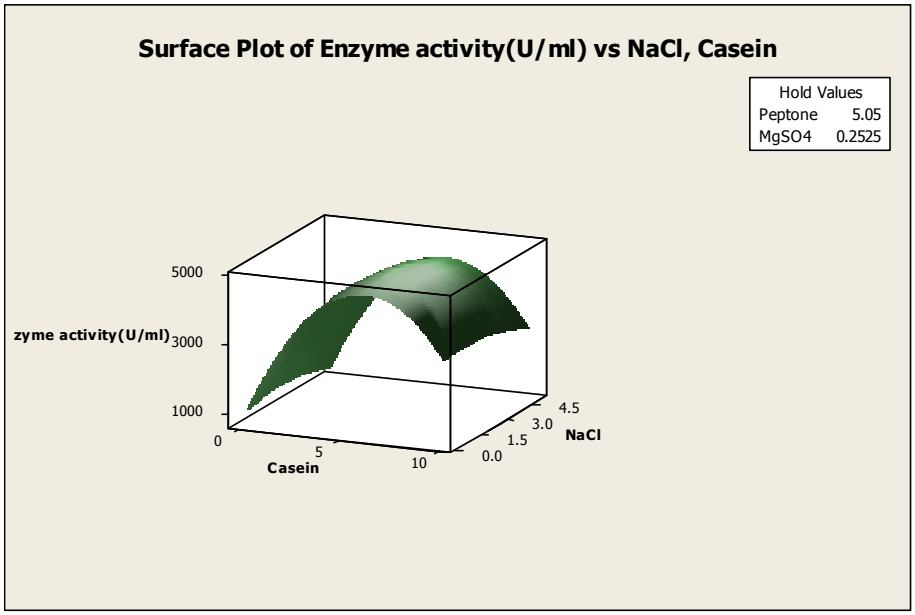

Fig. 3. Surface plot of enzyme activity vs NaCl and casein

Table 10. Solution for the model

Casein	Peptone	$MgSO_4$	NaCl	Predicted response	Obtained response
5.5 g	5.0 g	0.50 g	5.0 g	6379.9210 U/ml	6368.398 U/ml

5. CONCLUSION

It is extremely imperative to find out the new enzymes with novel properties for the improvement of human life. The present study has highlighted the isolation of a potent fibrinolytic enzyme producer and the screening of physical factors, various carbon, nitrogen and metal ions and optimization of media for the maximum production of the enzyme using statistical approaches. The isolate obtained was *Bacillus pumilus*, **ZR LS S2** and the study revealed that both physical parameters and media components affect the enzyme production by the isolate. Both the parameters were optimized by RSM and the obtained enzyme yield was four fold higher than unoptimized media. Also the organism obtained was novel, for the best of our knowledge, as there are no reports of fibrinolytic enzyme production by *Bacillus pumilus* **ZR LS S2**. Thus this paper introduces the application of RSM for the optimal production of fibrinolytic enzyme and introduces the isolate *Bacillus pumilus* **ZR LS S2** as a probable nominee for fibrinolytic enzyme production.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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