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LYSINIBACILLUS XYLANILYTICUS., A NOVEL BACTERIAL STRAIN ISOLATED FROM SOIL WITH FIBRINOLYTIC ACTIVITY

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ABSTRACT

A novel fibrinolytic enzyme producing bacteria was isolated and identified by morphological and biochemical characteristics and confirmed by 16S r DNA as *Lysinibacillus xylanilyticus.* The optimum conditions for enzyme production was determined by response surface methodology instead of one factor at a time method. Placket burman method was used for the initial screening process. In our study the newly isolated strain *Lysinbacillus xylanyliticus* was found to produce fibrinolytic enzyme (2468.713U/ml) using the statistically optimized media which contain glucose, soy peptone, magnesium sulphate and ammonium nitrate as components.

KEY WORDS: fibrinolytic enzyme, *Lysinibacillus xylanilyticus, response surface methodology*

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INTRODUCTION

Thrombosis is considered to be the cause of many common disorders, of which Cardio vascular disorders (CVD) occupy a pivotal position. At the beginning of the twentieth century, CVD's were responsible for fewer than 10% of the death worldwide. Today that figure is above 30% with approximately 80% of the burden occurring in the developing countries. It is predicted that by 2030 about 23.6 million people will die of CVD's[1]. Due to its prevalence CVD is likely to impose an ever mounting crash on our society emotionally, socially and economically. The major cause for this disorder is the disruption in the normal haemostatic mechanism which is tightly regulated in normal cases and functions in preventing disorders like thrombosis or bleeding[2,3]. The major protein involved in the formation of thrombi is the protein fibrin, which is formed from fibrinogen by the activity of the enzyme thrombin[4]. During the process of fibrinolysis the protein fibrin is digested into fibrin degradation products by the activity of plasmin. In normal conditions the formation and digestion of fibrin is well balanced; however when this balance is disrupted in favour of fibrin production, it will lead to the development of thromboses related disorders[5]. For the treatment and prevention of CVD's fibrinolytic enzymes are used, which act on the protein fibrin, thereby digesting the thrombi which is formed. Thrombolytic therapy is considered to be the best way to achieve the recanalization. Fibrinolytic enzymes are generally plasminogen activators such as tissue type plasminogen activator (t-PA), urokinase type plasminogen activator and the bacterial plasminogen activator, streptokinase[6]. Fibrinolytic enzymes have been isolated from various sources including microorganisms, snakes and polychaete worms[7]. Although used in a universal manner, these agents have undesirable side effects, exhibit a low specificity for fibrin and are relatively expensive. Therefore it is necessary to search for novel enzymes from other sources also[8]. While searching for microorganisms that produce fibrinolytic enzymes, a novel fermentation medium is of critical importance in the bioprocess improvement, because medium components can significantly affect the product yield and every organism is unique in its nutrient requirements for enzyme production[9]. The conventional 'one factor at a time' approach for media optimization is time consuming and fails to determine the interaction effect[10]. The statistical methods like Response Surface Methodology (RSM) are gaining worth currently in optimization studies. It involves a minimum number of experiments for a large number of factors. Successful application of RSM is used in optimization of various processes like bio fuel production, enzyme production including alkaline proteases and xylanases[11]. Because of the medicinal importance of the fibrinolytic enzyme and the fact that currently available agents possess significant shortcomings, this study aims the isolation & identification of potent fibrinolytic enzyme producing bacteria. Response surface methodology is applied for the optimization of the production media of the isolate.

MATERIALS AND METHODS

1. Isolation of protease producers.

Samples collected from different locations of Calicut district, Kerala were serially diluted and inoculated in casein broth, aseptically. After incubation at 48 hours, diluted samples were inoculated on casein agar plates. For qualitative determination of protease production, positive protease producers were selected, again spot inoculated on casein agar plates and zone of clearance was measured in each case.

2. Qualitative detection of fibrinolytic enzyme production by fibrin plate method.

Fibrin plate method of Astrup and Mullertz was used with slight modifications[12]. Fibrin plate was prepared by mixing Fibrinogen - 5ml (0.6% w/v) in 50 mM sodium phosphate buffer PH 7.4, with same volume of 2% w/v agar or agarose solution and 0.1 ml of thrombin (10NIH units/ml). For assessing protease activity, cultures were inoculated and observed for growth and clear zone around the colony. The colony with largest zone of clearance was selected for further activity.

3. Fibrinolytic assay.

1.4ml of Tris- HCl (50mM, PH 7.5) and 0.4ml of 0.72%(w/v) fibrinogen solution were taken in vials to which 0.1ml thrombin (20u/ml) was added and then kept in water bath at 37^{o}C (10 min). To this 0.1 ml of enzyme was added. The mixture was incubated at 37^{o}C for 1 hour and then 2ml of 0.2 molar TCA was added to it. Vials were kept for 20 minutes and centrifuged at 3000g for 5 min. The absorbance of the supernatant was measured at 280nm (UV Mini 1240,Shimadzu spectrophotometer) and converted to the amount of tyrosine equivalent. One unit of fibrinolytic activity was defined as the amount of enzyme releasing one micromole of tyrosine equivalent per hour[13].

4. Identification of the isolate by 16S r DNA method.

4.1. DNA isolation.

The DNA sample was isolated using the Samrook *et* al method[14]. The desiccated DNA samples were completely resuspended in 50µl of DNA dissolving buffer (TE buffer) and stored at -80^{o}C.

4.2. Polymerase Chain Reaction.

16S rDNA fragment was amplified by PCR from bacterial genomic DNA, using
16S rDNA universal primers:
10-30 F: 5'-GAG TTT GAT CCT GGC TCA G-3'
530R: 5'-G (AT) A TTA CCG CGG CGG CTG-3'
PCR was carried out in a final reaction volume of 25 µl in 200 µl capacity thin walled PCR tube. Composition of reaction mixture is Deionized water - 16.5µl, Taq buffer without $MgCl_2$ (10 X) - 2.5µl, $MgCl_2$ (15 mM) -1.0µl, dNTPs mix (10 mM each) - 1.5µl. 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 purified DNA, after determining its concentration, has been subjected for automated DNA sequencing. ABI3730xl Genetic Analyzer (Applied Bio systems, USA) has been used to carry out the sequencing.

5. Response surface methodology for the optimization of media

Response surface methodology (RSM) is a collection of statistical and mathematical techniques useful for developing, improving, and optimizing processes[15]. Placket Burman model (PBD) was used for the screening of factors which has significant effect on enzyme production[16]. Initially 16 factors were assessed using the PBD and 20 trial runs were carried out and four significant factors were selected for further optimization studies. A face centered central composite design (FCCCD) was applied to find out the significant levels of the independent variables and effect of their interactions on enzyme production. The independent variables selected for this study were glucose, peptone, ammonium nitrate and magnesium sulphate. The dependent variable selected was fibrinolytic enzyme activity (U/ml). Each independent variable was studied at 3 different levels; low, medium and high denoted as -1, 0 and +1. Each run was performed in triplicate and the average value for fibrinolytic activity was taken as the response for each run. A multiple regression was carried out to define the response in terms of independent variables and ANOVA was done to determine the statistical significance. Response surface plots were used to determine the interaction of different variables. The statistical software Minitab 14 was used for regression analysis of experimental data and to plot response surface, ANOVA was used to estimate the statistical parameters.

RESULTS AND DISCUSSION

It is indispensable to screen different sources for fibrinolytic enzymes as the currently used agents have significant shortcomings. In this study we have carried out isolation, identification and RSM based optimization of a potent fibrinolytic enzyme producer from soil.

1. ***Isolation of protease producers with fibrinolytic activity.***

Of the 70 isolates screened, 10 showed fibrinolytic activity on modified fibrin agar plate. Among these, the culture filtrate of strain B10 showed the highest fibrinolytic activity of 512U/ml and hence this strain was selected for further studies. Microorganisms were considered as an important source of

fibrinolytic enzymes and such enzymes were successfully discovered from different microorganisms[17].

2. Identification of the strain B10.

Based on morphological, physiological and biochemical characteristics, the strain was identified as *Lysinibacillus xylanilyticus* which is found to be a novel isolate, producing the fibrinolytic enzyme. To confirm the identification results further, the 16s rRNA gene sequence obtained was compared with sequences available in the gene bank. The isolate showed 99% similarity with *Lysinibacillus xylanilyticus.*

Consensus Sequence Data Culture B10: (556 bp)

GAGCTTGCTCCTTTGACGTTAGCGGCGGA
CGGGTGAGTAACACGTGGGCAACCTACCC
TATAGTTTGGGATAACTCCGGGAAACCGG
GGCTAATACCGAATAATCTCTTTTGCTTCAT
GGTGAAAGATTGAAAGACGGTTTCGGCTGT
CACTATAGGATGGGCCCGCGGCGCATTAG
CTAGTTGGTGAGGTAACGGCTCACCAAGG
CGACGATGCGTAGCCGACCTGAGAGGGTG
ATCGGCCACACTGGGACTGAGACACGGCC
CAGACTCCTACGGGAGGCAGCAGTAGGGA
ATCTTCCACAATGGGCGAAAGCCTGATGGA
GCAACGCCGCGTGAGTGAAGAAGGTTTTC
GGATCGTAAAACTCTGTTGTAAGGGAAGAA
CAAGTACAGTAGTAACTGGCTGTACCTTGA
CGGTACCTTATTAAAAAGCCACGGCTAACT
ACGTGCCAGCAGCCGCGGTAATACGTAGG
TGGCAAGCGTTGTCCGGAATTATTGGGCGT
AAAGCGCGCGCAGGCGGTCCTCTAAGTCT
GATGTGAAAGCCCACGGCTCAACCGT

2. ***Response surface methodology for the optimization of fibrinolytic enzyme.***

The conventional 'one factor at a time' method is time consuming, requires more experimental trials and also cannot predict the interaction effects[18], whereas the statistical methods for optimization can predict the interaction between different independent variables and it requires less number of experiments compared to the conventional method. The FCCCD design matrix of the variables, along with experimental and predicted values for enzyme activity, is shown in Table 1. The matrix was statistically analysed by ANOVA and the results are shown in tables 2.1 and 2.2. The ANOVA of the experimental model suggested that the model is highly significant as indicated by the high F value of 75.31. The lack of fit value (0.927) was insignificant and R^2 value is high (98.70) which indicates that the model could explain up to 98.70% of the variability of the response and the model did not explain 2.3% of the total variation. The linear coefficients A, C, D and the interaction coefficients BB, CC, AB, AD and BC were found to be significant. The quadratic coefficients B2 and C2 were also found to be significant. The calculated regression equation for the optimization of medium components showed fibrinolytic activity as a function of these variables. The RSM gave the following regression equation for fibrinolytic activity as a function of the independent variables A, B, C and D. The following equation was found to explain enzyme production. Y= 1255.84+ 259.52A+ 13.04B+ 76.45C - 126.28D -2.01A2+ 167.69B2 -148.96C2+ 62.49D2+ 163.08AB+ 29.58AC -68.58AD+ 257.70BC -106.20BD - 79.96CD-----(Eq.no.1), where Y represents the response and A, B, C and D the variables.Three dimensional response surface plots also show significant interaction effects between different independent variables. From the response surface plots, it is evident that the soy peptone has a significant effect on enzyme production. Enzyme yield is 2468.713U/ml in the optimized medium which is 2 fold higher than that in the unoptimized medium. Since the bioprocess technologies like bioremediation and biodegradation have emerged, many people are interested in statistical optimization of biological processes and productions by shortening time and increasing efficiencies[19]. In previous studies Liu etal[20] optimized fibrinolytic enzyme production using the media components peptone, glucose, calcium chloride and magnesium sulphate and obtained an activity of about 1300U/ml. In our study the newly isolated strain *Lysinbacillus xylanyliticus* was found to produce the enzyme with an activity of 2468.713U/ml using the statistically optimized media which contain glucose, soy peptone, magnesium sulphate and ammonium nitrate as components. In addition to establishing the optimal conditions for enzyme production the response surface

methodology also helps to predict the yield of the enzyme if the media composition is altered in some way using the derived quadratic equation (Eq.No.1). Thus the novel strain *Lysinibacillus xylanilyticus* can be considered as a suitable microorganism for large scale production of fibrinolyticenzyme.

TABLE 1

Experimental design and result of the central composite design.

RUN ORDER	FACTOR 1 GLUCOSE (%)	FACTOR 2SOY PEPTONE (%)	FACTOR 3$MgSO_4$ (%)	FACTOR4 $(NH_4)_2NO_3$ (%)	FIBRINOLYTIC ACTIVITY (U/ml)
1	20.0	5.5	0.25	0.25	1549.00
2	10.5	5.5	0.50	0.25	1245.00
3	10.5	5.5	0.00	0.25	998.34
4	10.5	5.5	0.25	0.25	1247.88
5	10.5	5.5	0.25	0.50	1225.34
6	10.5	10.0	0.25	0.25	1472.67
7	1.0	5.5	0.25	0.25	988.25
8	10.5	5.5	0.25	0.25	1247.88
9	10.5	1.0	0.25	0.25	1403.98
10	10.5	5.5	0.25	0.00	1440.91
11	20.0	10.0	0.50	0.50	1797.91
12	1.0	1.0	0.50	0.50	942.46
13	10.5	5.5	0.25	0.25	1247.88
14	1.0	1.0	0.00	0.00	1283.46
15	20.0	1.0	0.50	0.00	1400.30
16	20.0	1.0	0.00	0.50	1616.64
17	10.5	5.5	0.25	0.25	1247.88
18	20.0	10.0	0.00	0.50	1063.00
19	10.5	5.5	0.25	0.25	1247.88
20	20.0	1.0	0.00	0.00	1555.45
21	20.0	10.0	0.50	0.00	2468.71
22	1.0	10.0	0.50	0.50	946.38
23	20.0	10.0	0.00	0.00	1688.91
24	1.0	1.0	0.50	0.00	1105.86
25	1.0	10.0	0.50	0.00	1438.73
26	1.0	1.0	0.00	0.50	1548.37
27	20.0	1.0	0.50	0.50	1058.45
28	1.0	10.0	0.00	0.00	681.38
29	1.0	10.0	0.00	0.50	592.08
30	10.5	5.5	0.25	0.25	1247.88

TABLE 2.1

RESULTS OF THE REGRESSION ANALYSIS OF THE FCCCD

TERM	COEEFICIENT	SE COEFFICIENT	T	P
Constant	**1255.84**	**19.65**	**63.916**	**0.000**
Block	-10.24	14.86	-0.689	0.502
A – GLUCOSE	259.52	13.76	18.859	0.000
B-SOYPEPTONE	13.04	13.76	0.948	0.359
C-$MgSO_4$	76.45	13.76	5.556	0.000
D-$(NH_4)NO_3$	-126.28	13.76	9.177	0.000
AA	-2.01	13.76	0.055	0.957
BB	167.69	36.64	4.576	0.000
CC	-148.86	36.64	4.065	0.001
DD	62.49	36.64	1.705	0.110
AB	163.08	36.64	11.173	0.000
AC	29.58	36.64	2.026	0.062
AD	-68.58	14.60	-4.69	0.000
BC	257.70	14.60	17.656	0.000
BD	-106.20	14.60	7.276	0.000
CD	-79.26	14.60	-5.478	0.000

R-Sq = 98.7% R-Sq (adj) = 97.3%

TABLE 2.2
ANOVA FOR FCCCD

SOURCE	DF	SEQ SS	Adj SS	Adj MS	F	P
BLOCKS	1	4880	1618	1618	0.47	0.502
REGRESSION	14	3593742	3593742	256696	75.31	0.000
LINEAR	4	1607658	1607658	401914	117.91	0.000
SQUARE	4	126014	126014	31503	9.24	0.001
INTERACTION	6	1860071	1860071	310012	90.95	0.000
RESIDUAL ERROR	14	47722	47722	3409	-	-
LACK OF FIT	10	618429	618429	61843	0.33	0.927
PURE ERROR	4	0	0	0	-	-
TOTAL	29	3646344	-	-	-	-

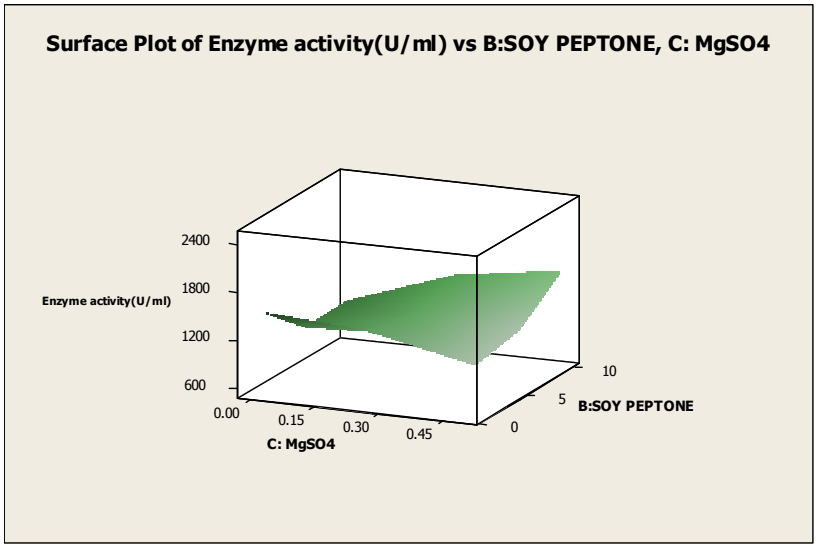

Figure.1
Response surface plot for fibrinolytic enzyme production by Lysinibacillus xylanilyticus, the interaction between a soy peptone and magnesium sulphate.

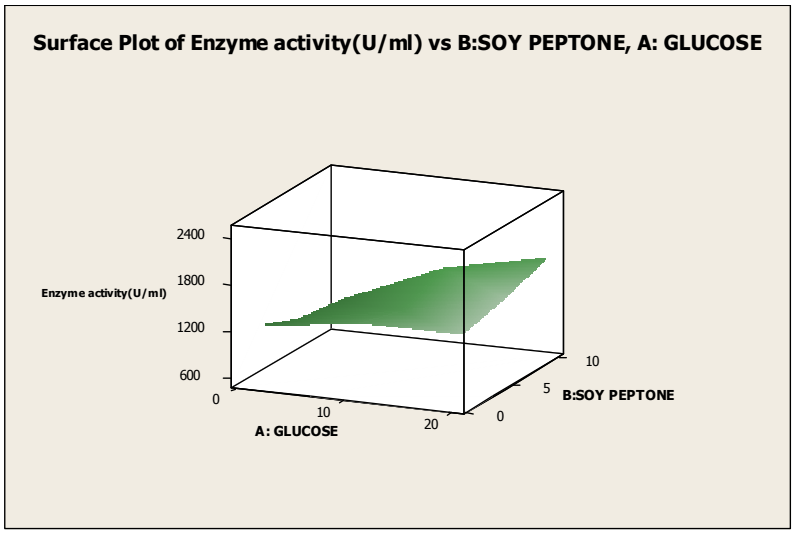

Figure.2
Response surface plot for fibrinolytic enzyme production by Lysinibacillus xylanilyticus, the interaction between Soy peptone and glucose.

CONCLUSION

The aim of the present study was the isolation and optimization of a fibrinolytic enzyme producer from soil. The isolate obtained in this study is a novel organism since there are no previous reports of the fibrinolytic potential of *Lysinibacillus xylanilyticus.* By using response surface methodology there was a two fold increase in the fibrinolytic enzyme production and further work is under progress for the purification of the enzyme from the same organism. Enzyme yield is 2468.713U/ml in the optimized medium, which is 2 fold higher than that in the unoptimized medium. The isolate, *Lysinibacillus xylanilyticus* may be considered as an industrially important enzyme producer and future studies will be focussed on the production of the fibrinolytic enzyme from this strain using cheap raw materials.

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