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Original article

Enhanced production of fibrinolytic enzyme by a new *Xanthomonas oryzae* IND3 using low-cost culture medium by response surface methodology

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ABSTRACT

Cardiovascular diseases (CVDs) cause high mortality throughout the world. Existing fibrinolytic agents are highly expensive and have many side effects. Microbial fibrinolytic enzymes are very much considered as novel therapeutic candidate for the treatment of CVDs. Reports on fibrinolytic enzyme from *Xanthomonas* sp. is lacking. This study reports fibrinolytic enzymes from *Xanthomonas oryzae* IND3 as it shows hyperactivity on fibrin-agarose plates. This organism utilized various agro-industrial wastes for enzymes production. Among all, cow dung enhanced more enzyme production, hence it was used as the low-cost substrate for statistical optimization of fibrinolytic protease in Solid state fermentation. Response surface methodology was employed to optimize the factors and enhanced yield by 4-fold. The interactions among the variables, viz, sucrose, yeast extract, and pH of the medium were investigated using Central Composite Design (CCD). The predicted fibrinolytic enzyme activity was 2340 U/g, and the observed fibrinolytic enzyme activity was 2294 ± 12.8 U/g. The fibrinolytic enzyme degraded blood clot *in vitro* completely. This study is the first report on statistical optimization of fibrinolytic enzyme production in SSF from *Xanthomonas* sp. The crude extract has immense activity on proteinaceous wastes. The production of fibrinolytic protease using the low-cost substrate could reduce the production cost of enzyme.

1. Introduction

Thrombosis is one of the important Cardiovascular diseases cause more deaths throughout the world. Thrombolytic agents such as streptokinase, tissue plasminogen activator (tPA) and urokinase plasminogen activator (u-PA) are commonly used to treat thrombosis. Although t-PA and u-PA are sate to treat thrombosis but every expensive. In the other hand, streptokinase cause serious side effects (Collen and Gold, 1990). In recent years, microbial fibrinolytic enzymes have been reported to treat and prevent CVDs. These enzymes have various therapeutic applications, including, oncolyticus, anticoagulants, thrombolytics and anti-inflammatories. There are various reports on fibrinolytic enzymes with thrombolytic applications from many sources, such as earth worm, snake venom, and fermented foods (Peng et al., 2003). Many studies involved the characterization of fibrinolytic enzyme, however, very few studies were carried on culture media optimization using statistical approach (Mukherjee and Rai, 2011). The fermentation media are the major cost determining factors for bacterial enzyme production, however the application of agro-residues could minimize the enzyme production cost and also valorization of agro-wastes to decrease environmental pollution (Bajaj et al., 2014). Many studies were conducted on agro-residues, such as pigeon pea (Johnvesly et al., 2002), potato peel (Mukherjee et al., 2008), apple pomace (Dhillon et al., 2012b), rice chaff (Tao et al., 1997), fish waste (Ramkumar et al., 2016), wheat bran (Almalki,

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2018) and agro-industrial wastes (Sadh et al., 2018) for the production of enzymes. These reported substrates were highly useful for the production of enzymes in solid-state fermentation (SSF).

Presently, *Bacillus* sp. and fungal isolates were very much exploited for fibrinolytic enzyme production. However, very fewer reports available on other bacterial species. The search for novel bacteria for bioprocess optimization for enzyme production is a continuous process. *Xanthomonas* sp. has the ability to produce various enzymes and was reported by various research groups. For example, *Xanthomonas axonopodis* pv. *punicae* strain has the ability to produce xylanolytic and cellulolytic enzymes (Amat et al., 2014). Lytic enzyme was produced from *Xanthomonas campestris* using low cost substrates (Da Silva et al., 2014). Kalashnikova et al. (2003) used three *Xanthomonas campestris* pv. *campestris* strains for their ability to produce proteolytic enzymes. However, the fibrinolytic enzyme production from the genus is highly limited. For industrial production of enzymes, a suitable culture medium is critical to enhance the yield for any selected isolates. Also, important prerequisites for commercial production are to reduce the cost of enzymes. This can be achieved by optimizing culture medium using agro-industrial residues. The conventional method of optimization is One-Factor-At-A-Time (OFAT) experiment, however this procedure is laborious and time consuming. Also, this method frequently fails to provide the interactive effect among the selected process parameters on product yield (Singh and Bajaj, 2016). Statistical optimization methods have several advantages over OVAT approach. A response surface methodology (RSM) has been widely used for the production of various enzymes, including nattokinase (Liu et al., 2005). Proteolytic enzymes have many applications in dairy-, meat-industries, and to hydrolyse proteinaceous discharges in aquatic environment. In municipal wastewater treatment, proteases hydrolyse proteins into smaller units (Cadoret et al., 2002). The therapeutic application of fibrinolytic protease is to dissolve the blood clot directly. The use of fibrinolytic proteases could avoid the use of highly toxic chemicals. The production of low-cost fibrinolytic enzyme is possible only by applying cheap agro-residues in SSF. The report on firinolytic protease production using low cost substrate by *Xanthomonas oryzae* is not available. The main objective of the present study is to optimize fibrinolytic enzyme from *Xathomonas oryzae* for biomedical applications.

2. Materials and methods

2.1. Screening and isolation of fibrinolytic protease producing bacteria

Rice was boiled for 60 min and allowed for microbial fermentation (32 ± 2 °C) for 48 h. It was further used for the screening of potent fibrinolytic protease producing bacteria. About one gram of rice was mixed with 100 ml sterile distilled water and plated on skimmed milk agar (Himedia, Mumbai, India) plates. Ten protease producing bacterial isolates were purified by standard method and further subjected for secondary screening. For secondary screening the bacterial isolates were cultured in submerged fermentation in nutrient broth and cell free extract was used for screening.

The protease producing bacteria were cultured in nutrient broth (Himedia, Mumbai, India) for 48 h at 37 ± 2 °C. After 48 h, fermented medium was centrifuged (5000*g* at 4 °C) for 15 min, and the supernatant was used for screening. The fibrinolytic protease activity was determined on fibrin-agarose plate (Astrup and Mullertz, 1952). In this plate, 2.5 ml fibrinogen (0.5%, w/v) and 100 μl thrombin (100 NIH U/ml) were incorporated. It was allowed to stand at 30 ± 2 °C for 1 h and 15-μl sample was dropped into wells. The plates were incubated at 30 ± 2 °C, and the fibrinolytic protease activity exhibited zone of hydrolysis around the well.

2.2. Molecular identification

The hyperactive fibrinolytic enzyme producing *X. oryzae* IND3 was cultured in the nutrient broth medium for 18 h at 37 ± 2 °C. The genomic DNA was extracted using a commercial kit (QIAGEN genomic DNA purification kit) according to the manufacturer's instructions. The16S rDNA gene was amplified using a PCR—Peltier Thermal Cycler Machine (USA). The amplified 16S rDNA was sequenced, and 857-bp16S rDNA gene sequences of the bacterial isolate were submitted to GenBank under the accession number: KF250419.

2.3. Solid state fermentation

In this study, banana peel, green gram husk, rice bran, wheat bran and cow dung was used as the substrate. About 5.0 g of these substrates were individually transferred in a 100-ml Erlenmeyer flask and moisture content was maintained at 90% (v/w) level using Tris-buffer (100 mM, pH 8.0). All Erlenmeyer flasks were sterilized and cooled. These flasks were inoculated individually with 0.5 ml of 18-h grown bacterial culture (OD 600 nm = 1.98 ± 0.240) and incubated at 37 ± 2 °C for 48 h in an incubator. After incubation, enzyme was extracted and the supernatant was used to screen the efficacy of substrate on enzyme production.

2.4. Fibrinolytic protease assay

The crude enzyme was mixed with Tris-HCl buffer (2.5 ml, 0.1 M, pH 7.8, 0.01 M calcium chloride) and fibrin (2.5 ml, 1.2%, w/v) and incubated at 37 °C for 30 min. The reaction was terminated by adding 5.0 ml of trichloroaceticacid (0.11 M), containing sodium acetate (0.22 M) and acetic acid (0.33 M). The absorbance of the sample was measured at 275 nm against blank (Ansen, 1938). Total protein estimation was carried out by the method of Lowry et al. (1951).

2.5. Screening of variables by one-variable-at-a-time approach

SSF was carried out as described previously. Cow dung which showed maximum enzyme production among the selected substrates was used as the substrate for optimization studies, until otherwise stated. The important nutrient factors, such as carbon source ([1%, w/w], glucose, sucrose, maltose, starch, and xylose), nitrogen source ([1%, w/w]; peptone, casein, yeast extract, gelatine, and urea), and inorganic ions ([1%, w/w], calcium chloride, magnesium chloride, sodium di-hydrogen phosphate, zinc sulphate, and mercury chloride), were screened.

2.6. Elucidation of process variables by a two-level full factorial design

A two-level full factorial design (2^5) was employed to screen the important factors affecting fibrinolytic protease production. The five selected factors were moisture, pH, sucrose (carbon source), yeast extract (nitrogen source), and sodium di-hydrogen phosphate (mineral salt). Each factor was analyzed at two different levels (Table 1a). The two-level full factorial design experiment was based on the first-order polynomial equation described below:

$$Y = \alpha_0 + \sum_i \alpha_i x_i + \sum_{ij} \alpha_{ij} x_i x_j + \sum_{ijk} \alpha_{ijk} x_i x_j x_k + \sum_{ijkl} \alpha_{ijkl} x_i x_j x_k x_l$$

where Y = fibrinolytic protease activity, αij = ij*th* interaction coefficient, αijk = ijk*th* interaction coefficient, and α0 = intercept

Table 1a
Factors involved according to a two-level full factorial design for optimization of fibrinolytic protease production.

Symbol	Variables	Units	Coded levels	
			−1	1
A	Moisture	%	80	100
B	pH		7	9
C	Sucrose	%	0.1	1
D	Yeast extract	%	0.1	1
E	NaH2PO4	%	0.01	1

Table 1b
Statistical analysis of 2^5 full factorial design showing values for each variables for fibrinolytic enzyme activity.

Run	Sucrose	Peptone	NaH_2PO_4	pH	Moisture	Enzyme
	A	B	C	D	E	activity (U/g)
1	−1	1	−1	1	−1	275
2	−1	1	1	1	1	540
3	1	1	1	1	−1	850
4	−1	−1	1	1	−1	385
5	−1	−1	−1	1	1	230
6	1	−1	1	−1	1	460
7	1	−1	1	1	−1	875
8	1	1	1	−1	−1	455
9	1	1	1	−1	1	1400
10	1	1	−1	1	−1	1000
11	1	−1	−1	−1	1	1190
12	−1	−1	−1	1	−1	1590
13	−1	1	−1	−1	1	495
14	−1	−1	1	1	1	700
15	−1	1	−1	−1	−1	2245
16	−1	1	1	−1	−1	260
17	1	−1	1	−1	−1	545
18	1	1	1	1	1	1220
19	1	−1	−1	1	−1	1525
20	−1	−1	−1	−1	−1	900
21	−1	1	−1	1	1	1325
22	1	−1	1	1	1	120
23	−1	−1	1	−1	−1	375
24	−1	−1	−1	−1	1	475
25	1	1	−1	−1	1	790
26	−1	1	1	−1	1	780
27	1	−1	−1	1	1	925
28	1	1	−1	−1	−1	505
29	−1	1	1	1	−1	510
30	−1	−1	1	−1	1	670
31	1	−1	−1	−1	−1	325
32	1	1	−1	1	1	680

Fibrinolytic protease assay was carried out in duplicate experimental runs, and the average value was taken as response "Y." Analysis of variance (ANOVA) was used to test the statistical significance and p-value < 0.05 was considered as statistically significant. The entire experimental setup for a two-level full factorial design for five variables is described in Table 1b. The most significant process parameters ($p < 0.05$) which influence on enzyme production were further optimized by RSM.

2.7. Central composite design

Central composite design (CCD) was frequently used to find the optimum concentration of variables. In our study, the selected factors, including, pH, sucrose, and yeast extract were employed at five levels (−α, −1, 0, +1, and +α) (Table 2a). For three variables, a total of 20 experiments run, including six center, six axial, and eight factorial points. SSF was carried out in duplicates as described earlier with the specific experimental matrix described in Table 2b. The results obtained were analyzed by ANOVA and the second-order polynomial equation is as follows.

$$Y = \alpha_0 + \alpha_1 A + \alpha_2 B + \alpha_3 C + \alpha_1\alpha_2 AB + \alpha_1\alpha_3 AC + \alpha_2\alpha_3 BC + \alpha_1\alpha_1 A^2 + \alpha_2\alpha_2 B^2 + \alpha_3\alpha_3 C^2$$

2.8. Application of fibrinolytic enzyme

2.8.1. Activity of fibrinolytic enzyme on blood clot in vitro

The Goat blood was collected from a slaughter house. Furthermore, the clot was washed several times with phosphate buffered saline (PBS) (pH 7.2) and cut into small pieces aseptically. The enzyme was diluted appropriately (200 U/ml) and incubated with blood clot (500 ± 50 mg) at 30 ± 2 °C. To the control vial, enzyme was not added with PBS. The tubes were incubated for 24 h and blood clot lysis was observed (Najafi et al., 2005).

2.8.2. Enzymatic hydrolysis of proteins from milk plant

Milk sewage hydrolysis experiment was carried out as described previously (Jung et al., 2002). Milk sewage was collected from Avin milk plant, Nagercoil, Tamilnadu, India. The protein hydrolysis was initiated by adding 200 U of crude fibrinolytic protease with milk sewage (100 ml) and incubated at room temperature (30 ± 2 °C) for 6 h. Total protein content was measured as described previously.

2.8.3. Hydrolysis of proteinaceous waste from sewage water

A liter of wastewater was collected at Nagercoil municipal area, Kanyakumari, Tamilnadu, India. The protein content of the wastewater was tested and concentrated by adding ammonium sulphate (70% saturation). The precipitated proteins were reconstituted at the concentration of 1 mg/ml and 0.1 ml enzyme solution was added to initiate the reaction. The reaction was terminated by 10% TCA (1 ml) solution after 0–60 min at a 15 min interval. Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) (12%) was used to monitor the hydrolysis proteins (Laemmli, 1970).

3. Results and discussion

3.1. Screening of fibrinolytic enzyme producing X. oryzae IND3

In our study, a clear zone was observed on the fibrin-agarose plate showing fibrinolytic enzyme activity of *X. oryzae* IND3 than other isolates (Fig. 1). Many reports have demonstrated the production of proteases from various *Xanthomonas* sp., including, *Xanthomonas maltophilia* (Margesin and Schinner, 1991), *Xanthomonas campestris* pv. zinniae (Sun, 1991), *Xanthomonas* sp. (Silva et al., 2014), and *Xanthomonas campestris* (Dow et al., 1993). Recently, proteolytic enzyme was characterized from *Xanthomonas campestris* pv. *vesicatoria* by Sole et al. (2015). However, production of fibrinolytic enzyme from *X. oryzae* was not reported. Also, statistical optimization of fibrinolytic enzyme production other than *Bacillus* sp. is highly limited.

3.2. Valorization of agro-residues for enzyme bioprocess

In the present study, agro residues were screened for the production of fibrinolytic enzyme from *X. oryzae* IND3. All selected substrates supported fibrinolytic enzyme production. However, the enzyme yield varied widely. The enzyme yield was 132 ± 12.1, 334 ± 8.4, 217 ± 6.9, 402 ± 11.3, and 419 ± 9.8 U/g for the substrates banana peel, green gram husk, rice bran, wheat bran and cow dung, respectively. Previously, various wastes were utilized for the production of proteolytic enzymes. The proteinaceous tannery wastes were utilized as the cheap substrate by Ravindran et al. (2011). Considering the availability of substrate, cow dung is

Table 1c
Analysis of variance for 2^5 factorial experimental design.

Source	Sum of squares	df	Mean square	*F*-value	*p*-value	
Model	6.952 + 006	29	2.40E+05	92.54	0.0107	Significant
A-Moisture	3.85E+04	1	3.85E+04	14.86	0.0612	
B-pH	1.30E+05	1	1.30E+05	50.2	0.0193	
C-Sucrose	5.86E+05	1	5.86E+05	226.16	0.0044	
Yeast extract	2.42E+04	1	2.42E+04	9.34	0.0924	
E- NaH2PO4	1.20E+04	1	1.20E+04	4.64	0.1644	
AC	1.65E+05	1	1.65E+05	63.81	0.0153	
AD	1.47E+05	1	1.47E+05	56.8	0.0172	
AE	1.29E+05	1	1.29E+05	49.71	0.0195	
BC	9.35E+04	1	9.35E+04	36.1	0.0266	
BD	1.18E+05	1	1.18E+05	45.4	0.0213	
BE	2.59E+05	1	2.59E+05	100.05	0.0098	
CE	4.73E+05	1	4.73E+05	182.53	0.0054	
DE	1.15E+05	1	1.15E+05	44.47	0.0218	
ABC	5.25E+05	1	5.25E+05	202.77	0.0049	
ABD	5.20E+04	1	5.20E+04	20.07	0.0464	
ABE	2.15E+04	1	2.15E+04	8.31	0.1022	
ACD	1.08E+05	1	1.08E+05	41.73	0.0231	
ACE	3.61E+05	1	3.61E+05	139.45	0.0071	
ADE	6.93E+05	1	6.93E+05	267.6	0.0037	
BCD	1.70E+05	1	1.70E+05	65.49	0.0149	
BCE	5.36E+04	1	5.36E+04	20.7	0.0451	
BDE	5.46E+05	1	5.46E+05	210.76	0.0047	
CDE	7.13E+04	1	7.13E+04	27.5	0.0345	
ABCD	1.90E+04	1	1.90E+04	7.34	0.1135	
ABCE	4.05E+05	1	4.05E+05	156.33	0.0063	
ABDE	1.61E+05	1	1.61E+05	62.16	0.0157	
ACDE	3.12E+05	1	3.12E+05	120.45	0.0082	
BCDE	7.84E+05	1	7.84E+05	302.78	0.0033	
ABCDE	3.79E+05	1	3.79E+05	146.08	0.0068	
Residual	5.18E+03	2	2.59E+03			
Cor Total	6.96E+06	31				

Table 2a
Range of media components for the production of fibrinolytic protease.

Variables	Symbol	Coded values				
		−α	−1	0	1	+α
pH	*A*	6.32	7	8	9	9.68
Sucrose	*B*	−0.21	0.1	0.55	1	1.31
Yeast extract	*C*	−0.21	0.1	0.55	1	1.31

Table 2b
Central composite design matrix for the production of fibrinolytic protease from *X. oryzae*.

Std	*A*:pH	*B*:Sucrose	*C*:Yeast extract	Enzyme activity (U/g)
1	−1	1	1	622
2	1	1	1	1590
3	0	0	0	1269
4	1	1	−1	2068
5	1.682	0	0	1050
6	0	0	0	1231
7	0	0	0	1290
8	0	0	0	1249
9	0	1.682	1	1500
10	0	0	1.682	1508
11	−1	1	−1	841
12	−1	−1	1	2213
13	1	−1	−1	873
14	0	0	0	1348
15	−1	−1	−1	1372
16	0	−1.682	0	2110
17	0	0	−1.682	1200
18	−1.682	0	0	810
19	0	0	0	1308
20	1	−1	1	1480

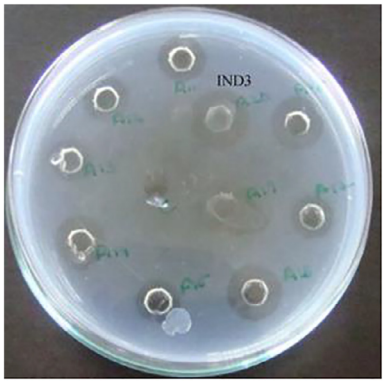

Fig. 1. Fibrinolytic enzyme activity of the selected bacterial isolates.

available widely and is too cheap than other reported substrates elsewhere. The application of cow dung as the substrate for the production of enzyme in an industrial scale could reduce the environmental pollution. This is the first report on the production of fibrinolytic protease by *Xanthomonas* sp. using low cost substrate

in SSF and optimization by statistical approach for industrial processes. Previously, cow dung was utilized for the production of fibrinolytic enzyme by *Bacillus* sp. IND7 and *Paenibacillus* sp. IND8 (Vijayaraghavan et al., 2016; Vijayaraghavan and Vincent, 2014b).

3.3. Optimization of fibrinolytic enzyme production by OVAT approach

The screened *X. oryzae* IND3 utilized various carbon sources and these carbon sources induced enzyme production. Enzyme production was high when sucrose was used as the carbon source (481 ± 16.9 U/g) (Fig. 2a) and low in the substrate containing glucose. This low enzyme activity could be the result of catalytic repression. It was previously reported that the increased production of proteolytic enzymes by the supplementation of maltose (Tsuchiya et al., 1991), and sucrose (Phadatare et al., 1993) along with the major components. The complex nitrogen sources were previously reported to enhance the production of fibrinolytic enzymes. In our study, fibrinolytic protease yield was found to be high when yeast extract was supplemented with substrate (597 ± 12.6 U/g) (Fig. 2b). The present study revealed that yeast extract was found to be the appropriate nitrogen source for enzyme production. This result was in accordance with result on fibrinolytic protease production by *Proteus penneri* SP-20 (Jhample et al., 2015).

In the present study, supplementation of sodium dihydrogen phosphate (1%, w/w) with substrate enhanced the production of fibrinolytic proteases (548 ± 9.64 U/g) than control (Fig. 2c). Supplementation of sodium dihydrogen phosphate could responsible for buffering the culture medium. This could be the reason for the increased fibrinolytic activity in the culture medium in the presence of this salt. This result was in accordance the observations made earlier with *Paenibacillus* sp. IND8 (Vijayaraghavan and Vincent, 2014b). It was also described that the mineral salts, such as calcium, copper, cobalt, manganese, and magnesium, were very much required for the production of proteolytic enzymes. Moreover, the requirement of mineral salts for the production of proteolytic enzymes varies widely, for example, potassium phosphate enhanced fibrinolytic enzyme production in *Bacillus licheniformis* (Mao et al., 1992).

3.4. Optimization of fibrinolytic protease production by two level full factorial designs

A two-level (2^5) full factorial design was used to screen all selected variables (moisture, pH, sucrose, yeast extract, and sodium dihydrogen phosphate) for fibrinolytic protease production. The response (Y) for two level full factorial design is shown in Table 1b. The two-level full factorial results revealed a wide variation on fibrinolytic enzyme yield. The designed 2 level full factorial statistical model was significant ($p < 0.05$). In this model, pH, sucrose, and yeast extract were most significant factors ($p < 0.05$). The first-order regression equation showed fibrinolytic protease activity, as a function of moisture, pH, sucrose, yeast extract, and sodium dihydrogen phosphate in terms of coded factors:

$$\begin{aligned}\text{Enzyme activity} = {} & +769.38 + 34.69A + 63.75B - 135.31C \\ & + 27.50D - 19.38E + 71.87AC + 67.81AD + 53.44AE \\ & + 54.06BC - 60.63BD + 90.00BE + 121.56CE - 60.00DE \\ & + 128.13ABC + 40.31ABD \\ & + 25.94ABE - 58.12ACD - 106.25ACE - 147.19ADE \\ & + 72.81BCD + 40.94BCE \\ & + 130.63BDE - 47.19CDE - 24.37ABCD \\ & + 112.50ABCE - 70.94ABDE \\ & + 98.75ACDE - 156.56BCDE + 108.75ABCDE\end{aligned}$$

where *Y* is the fibrinolytic protease activity (U/g), A, B, C, D, and E are moisture, pH, sucrose, yeast extract, and sodium di hydrogen phosphate, respectively.

The interaction between AC, AD, AE, BC, BE, CE, ABC, ABD, ABE, BCE, BDE, ABCE, ACDE, and ABCD had a positive interactive effect on enzyme production. The lack of fit of this model was non-significant, which confirmed that the designed model is good. According to the results, 80% moisture, pH 9.0, 0.1% sucrose, 0.1% yeast extract, and 0.1% NaH_2PO_4 significantly enhanced fibrinolytic protease production (2245 U/g) (Table 1c). Moisture content is one of the significant factors in SSF. However, in our study, the moisture content of the medium was not statistically significant ($p > 0.05$). Unlike other substrates, cow dung possesses high moisture holding capacity, and it supports the anchorage of bacterial cells in a wide range of moisture content. This could be the reason for non-significant moisture level in this experiment. This indicated that *X. oryzae* can produce enzyme at wide moisture content. The predicted R^2 of this model was 0.8094, which was in reasonable agreement with the R^2 of this model (0.993), and the adjusted R^2 of this model was 0.988.

3.5. Central composite design and response surface methodology

The concentrations of sucrose, yeast extract, and pH value were chosen for optimizing medium composition. Table 2b shows the experimental result of CCD. The designed model was well fit to the quadratic response surface model. The following regression equation of the second-order model provides the levels of fibrinolytic protease activity as a function of sucrose, yeast extract, and pH.

$$\begin{aligned}\text{Enzyme activity} = {} & +1282.03 + 100.01A - 134.94B + 92.92C \\ & + 428.38AB - 61.62AC - 268.12BC - 121.57A^2 + 187.79B^2 \\ & + 28.34C^2\end{aligned}$$

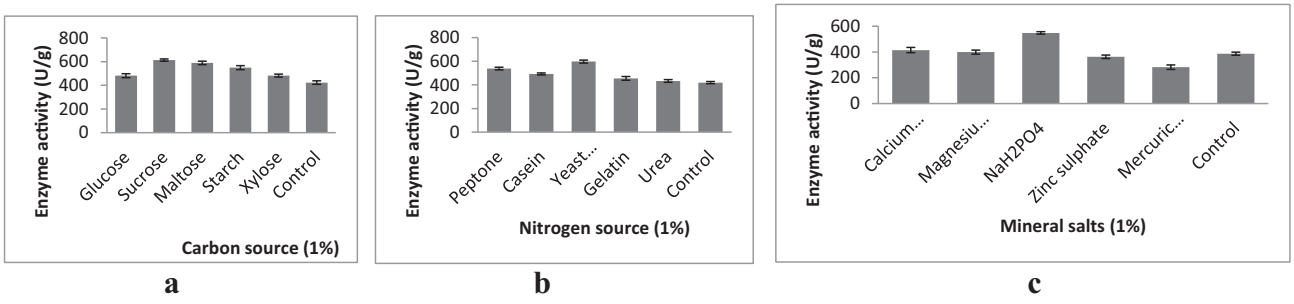

Fig. 2. Effect of carbon source (a), nitrogen source (b) and mineral salts (c) on fibrinolytic protease production.

Table 2c
Analysis of variance for central composite design.

Source	Sum of Squares	df	Mean Square	F-Value	p-Value	
Model	3.38E+06	9	3.75E+05	97.55	<0.0001	Significant
A-pH	1.37E+05	1	1.37E+05	35.54	0.0001	
B-Sucrose	2.49E+05	1	2.49E+05	64.62	<0.0001	
C-Yeast extract	1.18E+05	1	1.18E+05	30.64	0.0002	
AB	1.47E+06	1	1.47E+06	381.46	<0.0001	
AC	3.04E+04	1	3.04E+04	7.89	0.0185	
BC	5.75E+05	1	5.75E+05	149.44	<0.0001	
A^2	2.13E+05	1	2.13E+05	55.34	<0.0001	
B^2	5.08E+05	1	5.08E+05	132.06	<0.0001	
C^2	1.16E+04	1	1.16E+04	3.01	0.1136	
Residual	3.85E+04	10	3848.45			
Lack of fit	29530.99	5	5906.2	3.3	0.1081	Not significant
Pure error	8.95E+03	5	1790.7			
Cor total	3.42E+06	19				

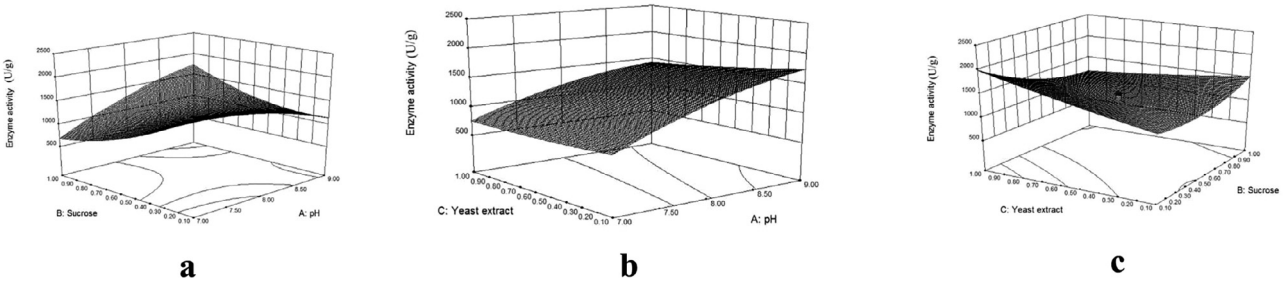

Fig. 3. Response surface plot showing the effect of pH and sucrose concentration and their interactive effect on the production of fibrinolytic enzyme (a). Response surface plot showing the effect of pH and yeast extract concentration and their interactive effect on the production of fibrinolytic enzyme (b). Response surface plot showing the effect of sucrose and yeast extract concentration and their interactive effect on the production of fibrinolytic enzyme (c).

where *Y* is the fibrinolytic activity (U/g), and A, B, and C were sucrose, yeast extract, and pH, respectively.

The F-value of the CCD model was 97.55, and the corresponding *p*-value was <0.001 (Table 2c). In this model, *p*-value was < 0.05 for the variables pH, sucrose, and yeast extract. These suggested that the concentration of sucrose, yeast extract, and pH of the culture medium has significant impact on fibrinolytic enzyme production. The *p*-value of the lack of fit was 0.1081, which revealed that the lack of fit of this model was non-significant. Non-significant lack of fit is good. The coefficient of determination (R^2) for this model was 0.9887. 3D response surface curves explained the interactive effects of variables on fibrinolytic enzyme production (Fig. 3a–c). The optimal levels of the important variables for the maximum fibrinolytic protease production for *A. oryzae* IND3 were as follows: pH 9.0, 0.96% sucrose, and 0.1% yeast extract. Fibrinolytic enzyme production was 2293 ± 12.8 U/g at these optimized concentration. The observed result (2293 ± 12.8 U/g) was close with the predicted response (2340 U/g). Results revealed that the developed model was very highly accurate and reliable for predicting the response.

In industrial point of view, a statistical experimental design played a significant role in enzyme production. RSM is one of the valid such statistical tools for studying the interactive effect of variables and to determine the optimal concentration of selected factors for enzyme production. RSM allows rapid screening of variables with minimum effect. In RSM, 3D helps simple visualization on interactions among variables (Mullai et al., 2010). RSM has been used for the production of fibrinolytic enzyme from *Bacillus cereus* IND1 and *Bacillus natto* NLSSE using CCD and RSM (Vijayaraghavan and Vincent, 2014a; Liu et al., 2005). In this study, the designed CCD model was effective and found to be suitable to enhance the production of protease. After statistical approach, fibrinolytic protease yield was 4-fold higher than un-optimized medium.

3.6. Applications of fibrinolytic enzymes

In our study, fibrinolytic enzyme effectively hydrolyzed blood clot directly *invitro*. The blood clot digestion was 3.8 ± 2.1, 17.8 ± 3.2, 39 ± 3.2, 82.5 ± 3.8, 97 ± 2.9 and 100 ± 2.1% after 2, 4, 6, 8, 10 and 12 h, respectively. In our study, after 12-h incubation, the fibrinolytic enzyme digested blood clot completely and converted blood clot into soluble form. Najafi et al. (2005) previously used crude protease and studied blood clot lytic activity *in vitro* from *Pseudomonas aeruginosa* PD100. Because of its blood clot lytic activity of enzyme from *X. oryzae* IND3, this enzyme may have great applications to treat thrombosis. Europe and Asia are the two major consumers of meat by-products, including lamb and beef. These industries generate blood wastes and in recent years increased attentions have been made to utilize these wastes (Jayathilakan et al., 2012). Also, the application of protease on removal of blood clot was studied by Anwar and Saleemuddin, 1997). In our study, the proteolytic enzyme hydrolyzed waste milk and protein content of waste milk sample was initially 30.02 ± 0.54 mg/ml. About the application of enzyme, it hydrolyzed the available proteins. Results revealed that the milk proteins concentration decreased as 21.07 ± 0.31, 18.3 ± 0.94, 14.8 ± 0.26, 6.03 ± 0.09, 1.29 ± 0.41, and 0.003 ± 0.001 mg/ml, after 10, 20, 30, 40, 50, and 60 min, respectively. In the present study, crude enzyme was subjected to digest proteins from sewage water. The protein content of municipal wastewater was 64.8 ± 0.063 mg/ml. The protein content was decreased as 49 ± 0.38, 37.1 ± 0.29, 23.8 ± 0.27, 1.92 ± 0.16, and 0.027 ± 0.001 mg/ml after, 10, 20, 30, 40, and 50 min incubation with enzyme. After 60 min incubation, the fibrinolytic enzyme digested considerable amount of protein from sewage water. The SDS-PAGE analysis revealed decreased protein content from municipal waste at higher incubation time

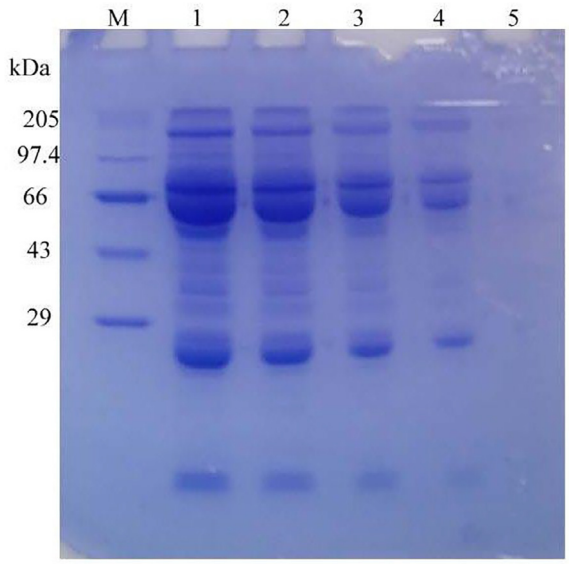

Fig. 4. SDS–PAGE results of solubilization of proteins from the municipal wastewater (M: protein marker; Lane 1: protein profile of crude protein precipitated from slaughterhouse wastewater without incubation with enzyme (control); Lane 2: crude protein profile after 15 min digestion with enzyme; Lane 3: digestion after 30 min; Lane 4: digestion after 45 min; Lane 5: digestion after 60 min).

with enzyme (Fig. 4). The extracellular microbial enzymes could hydrolyze proteins into smaller units (Codoret et al., 2002).

4. Conclusions

Fibrinolytic enzyme has potent application to treat cardiovascular diseases. This study reports the optimization of fibrinolytic protease by *Xanthomonas oryzae* IND3 using statistical approach in solid state fermentation. It completely digested Goat blood clot invitro after 6 h. Also, the crude extracellular enzyme effectively solubilizes proteins from waste milk from the processing plant and protein waste from municipal wastewater. Considering the efficiency of *X. oryzae* IND3 fibrinolytic enzyme, this enzyme could be useful in the treatment of cardiovascular diseases.

Conflict of Interest Statement

The authors declared no conflict of interest.

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