Biotechnology Reports 7 (2015) 135–142

Contents lists available at ScienceDirect

Biotechnology Reports

journal homepage: www.elsevier.com/locate/btre

A low cost fermentation medium for potential fibrinolytic enzyme production by a newly isolated marine bacterium, *Shewanella* sp. IND20☆

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ARTICLE INFO

Article history:
Received 6 March 2015
Received in revised form 20 May 2015
Accepted 25 June 2015
Available online 30 June 2015

Keywords:
Fibrinolytic enzyme
Shewanella sp.
Optimization
Thrombolytic agent
Response surface methodology

ABSTRACT

Agro-residues were used as the substrate for the production of fibrinolytic enzyme in solid state fermentation. In this study, two-level full factorial design (2^5) and response surface methodology were applied to optimize a fermentation medium for the production of fibrinolytic enzyme from the marine isolate *Shewanella* sp. IND20. The 2^5 factorial design demonstrated that the physical factors (pH and moisture) and nutrient factors (trehalose, casein, and sodium dihydrogen phosphate) had significant effect on fibrinolytic enzyme production. Central composite design was employed to search for the optimal concentration of the three factors, namely moisture, pH, and trehalose, and the experimental results were fitted with a second-order polynomial model at 99% level ($p < 0.0001$). The optimized medium showed 2751 U/mL of fibrinolytic activity, which was 2.5-fold higher than unoptimized medium. The molecular weight of fibrinolytic enzyme was found to be 55.5 kDa. The optimum pH and temperature were 8.0 and 50 °C, respectively.

1. Introduction

Cardiovascular diseases (CVDs) such as acute myocardial infarction and ischemic heart diseases are the leading cause of death in world wide. Among the different types of CVDs, thrombosis is one of the most widely occurring diseases in modern life. One of the major pharmaceutical applications of microbial fibrinolytic proteases is in the treatment of thrombosis [24]. A variety of fibrinolytic enzymes such as tissue plasminogen activator (t-PA), urokinase plasminogen activator (u-PA), and bacterial PA (streptokinase) have been extensively studied and used as thrombolytic agents [25]. However, these agents have some limitations such as excessive cost of clinical applications, undesirable side effects such as excessive bleeding and recurrence at the site of the residual thrombosis [7], difficulty in long-term use, low specificity to fibrin in the cases of u-PA and streptokinase, and short half-life in the cases of t-PA and u-PA. Hence, several lines of investigations are currently being pursued to enhance the efficacy and specificity of fibrinolytic therapy [24].

Nattokinase, a potent bacterial fibrinolytic enzyme was isolated from the traditional Japanese fermented food "*natto*" [33]. After the discovery of nattokinase, the fibrinolytic enzyme-producing microbes have been reported, which include *Staphylococcus aureus* [3,21],*Bacillus subtilis* A26 [1] and *B. subtilis* TKU007 [36]. The marine microorganisms provide an interesting alternative for therapeutic molecules. Enzymes produced by marine microorganisms can provide numerous advantages over traditional enzymes due to the activities at wide pH and temperature ranges [19] and can catalyze various biochemical reactions [23]. Many marine microorganisms were subjected for the production and characterization of fibrinolytic enzymes [18,23]. These fibrinolytic enzymes have potential for fortification and nutraceutical applications, such that their use could effectively prevent CVDs [24]. The protease secreting *Shewanella* sp. strain Ac10 [20] was explored but no fibrinolytic enzyme production was reported from the genus *Shewanella*.

Although many fibrinolytic enzymes have been purified and characterized; only very few reports are available concerning culture medium optimization by statistical approach [22]. Statistically designed experiments have many advantages over traditional one-at-a-time optimization methods. Response surface methodology (RSM) is a well-known statistical method applied in

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http://dx.doi.org/10.1016/j.btre.2015.06.005

the optimization of medium constituents and other critical variables responsible for the production of enzymes. The statistical experimental designs such as two-level full factorial design (2^5) and RSM involve a minimum number of experiments for a large number of factors, by which improvement in enzyme production has been demonstrated [13]. In enzyme bioprocess, designing a suitable medium for maximum production is critically important because the medium components significantly affect the product yield.

In Solid state fermentation (SSF), availability of medium components and cost of the substrate are the critical factors in an industrial point of view. Recently, cow dung has been reported as one of the cheap solid substrates for the production of proteolytic enzymes by *Bacillus* sp. and *Halomonas* sp. [34,35]. Interestingly, its availability is high than the reported solid substrates. Very few studies were reported on fibrinolytic enzyme production using statistical methods in SSF. This article reports the production of fibrinolytic enzymes in solid-state culture by a marine isolate, *Shewanella* sp. using agro-residues. The aim of the present study is to optimize the fibrinolytic enzyme production by a marine isolate, *Shewanella* sp. using RSM.

2. Materials and methods

2.1. Screening of a fibrinolytic enzyme-producing isolate

A fibrinolytic enzyme-producing bacterial isolate IND20 was isolated from the fish *Sardinella longiceps*. Fishes were collected from the Kanyakumari fish landing centre (8.0780°N, 77.5410°E, South West coast, Tamilnadu, India) and kept in ice immediately. The scales were carefully removed by forceps, homogenized and plated on skimmed milk agar plates (peptone, 5 g/L; beef extract, 1.5 g/L; yeast extract, 1.5 g/L; sodium chloride, 35 g/L; agar, 15 g/L; skim milk, 10 g/L) and incubated at 37 °C for 24–48 h. A clear zone on skimmed milk agar plates gave an indication of protease-producing isolates. Protease-producing organisms were further cultured in nutrient broth medium (peptone, 5 g/L; beef extract, 1.5 g/L; yeast extract, 1.5 g/L; sodium chloride, 35 g/L; and casein, 10 g/L) and incubated at 37 °C for 24–48 h in a shaker (150 rpm). After 48 h, the cultures were centrifuged at 10,000 rpm for 10 min at 4 °C, and the supernatant was used to screen fibrinolytic activity. Fibrinolytic activity of the crude enzyme was tested in a fibrin plate composed of 0.1 M sodium phosphate buffer (pH 7.4), 1% agarose (w/v), 0.5% fibrinogen (w/v), and thrombin (100 NIH U/mL) [5]. The fibrin plate was allowed to stand for 1 h at room temperature to form a fibrin clot layer. Ten microliters of crude enzyme was dropped into holes and incubated at 37 °C for 5 h. The fibrinolytic enzyme exhibited a clear zone of degradation of fibrin around the well, thus indicating its fibrinolytic activity. Isolates that formed a clear zone around their wells were selected, and IND20 was retained for this study.

2.2. 16S rDNA sequencing

The genomic DNA was extracted from the cells of an 18-h culture using QIAGEN genomic DNA purification kit according to the manufacturer's instructions. The 16S rDNA gene was amplified by PCR (Peltier Thermal Cycler Machine, USA) using the upstream (P1: 5′-AGAGTTTGATCMTGGCTAG-3′) and the downstream primers (P2: 5′-ACGGGCGGTGTGTRC-3′) and DNA polymerase. The amplified product was sequenced, and sequence comparison with the databases was performed using BLAST through the NCBI server [2].

2.3. Fibrinolytic enzyme assay

The culture supernatant (0.1 mL) was mixed with 2.5 mL of 0.1 M Tris–HCl buffer (pH 7.8) containing 0.01 M calcium chloride. To this, 2.5 mL of fibrin suspension (1.2%, w/v) was added and incubated at 37 °C for 15 min. The reaction was stopped by adding 5.0 mL of 0.11 M trichloroacetic acid containing 0.22 M sodium acetate and 0.33 M acetic acid. The absorbance was measured at 275 nm against sample blank. A standard curve was performed using L-tyrosine. One unit of fibrinolytic activity was defined as the amount of enzyme that liberates 1 μg of tyrosine per minute under the experimental conditions used.

2.4. Substrates

The substrates namely, banana peel, cow dung, rice bran, wheat bran and green gram husk were dried for several days. These were powdered using a mixer grinder, sieved, and stored in airtight containers before further use.

2.5. Inoculum

A loopful culture of bacterial isolate IND20 was inoculated in the nutrient broth medium (peptone, 5 g/L; beef extract, 1.5 g/L; yeast extract, 1.5 g/L; and sodium chloride, 35 g/L) and incubated at 37 °C for 18 h. It was stored at 2–8 °C for further studies.

2.6. Elucidation of agro-residues for fibrinolytic enzyme production

About 2.0 g of agro-residues (banana peel, cow dung, rice bran, wheat bran and green gram husk) were taken in an Erlenmeyer flask and moistened with 2 mL 0.1 M Tris–HCl buffer (pH 8.0). To these flasks, 0.2 mL of 18 h grown *Shewanella* sp. IND20 was inoculated and incubated at 37 °C for 72 h. The enzyme was extracted from the fermented medium as described previously [35]. This was centrifuged at 10,000 × *g* for 10 min at 4 °C, and the clear supernatant was used as the crude enzyme. All experiments were carried out in duplicates, and the average value was reported.

2.7. Screening of nutrient and physical factors by one-factor-at-a-time approach

Cow dung was used as the substrate for the production of fibrinolytic enzyme in SSF. SSF was carried out separately in a 100-mL Erlenmeyer flask containing 2.0 g (w/w) of the substrate (cow dung) moistened with 2.0 mL Tris–HCl buffer (0.1 M, pH 8.0). The contents were sterilized and inoculated with 0.2 mL of 18 h grown culture broth (1.239 OD at 600 nm) under sterile conditions. The process parameters such as the fermentation period (24–96 h), pH (6.0–10.0), moisture content (60–140%), inoculum size (3–15%), carbon sources (1%, w/w; maltose, sucrose, starch, glucose, xylose, and trehalose), nitrogen sources (1%, w/w; casein, yeast extract, peptone, beef extract, gelatin, and urea), and inorganic salts (0.1%, w/w; ammonium chloride, sodium dihydrogen phosphate (NaH_2PO_4), calcium chloride, sodium nitrate, disodium hydrogen phosphate (Na_2HPO_4), ammonium sulphate, and ferrous sulphate) were evaluated. The enzyme was extracted from the fermented medium as described previously.

2.8. Identification of the significant variables using the 2^5 factorial design

The 2^5 factorial design is one of the efficient methods to screen out the important nutrient and physiochemical parameters from a large number of process variables affecting fibrinolytic enzyme production. Five significant factors such as trehalose (carbon source), casein (nitrogen source), NaH_2PO_4 (inorganic salt), pH, and moisture content of the medium were selected for the analysis. The ranges of variables were fixed on the basis of first-step optimization. The 2^5 factorial design allows the evaluation of N (5) variables

in N (32) experiments; each variable was examined at two levels: −1 for a low level and +1 for a high level (Table 1a). The other factors such as fermentation period and inoculum level were kept at optimum level. The 2^5 factorial design was based on the following first-order polynomial model:

$$Y = \alpha_0 + \sum_i \alpha_i x_i + \sum_{ij} \alpha_{ij} x_i x_j + \sum_{ijk} \alpha_{ijk} x_i x_j x_k + \sum_{ijkl} \alpha_{ijkl} x_i x_j x_k x_l + \sum_{ijklm} \alpha_{ijklm} x_i x_j x_k x_l x_m \quad (1)$$

where Y is the response (fibrinolytic activity); α_{ij}, α_{ijk}, α_{ijkl}, and α_{ijklm} were the ijth, ijkth, $ijkl$th, and $ijklm$th interaction coefficients, respectively; α_i was the ith linear coefficient; and α_0 was an intercept.

Fibrinolytic activity assay was carried out in triplicates, and the averages of these experimental values were taken as response Y (Table 1b). Analysis of variance (ANOVA) was used to estimate the statistical parameters, and the values of "Prob > F" less than 0.05 indicate that the model terms are significant (Table 2). Statistical software, Design-Expert 8.0.7.0 (Stat-Ease Inc., Minneapolis, USA), was used to analyze the experimental 2^5 factorial design. The significant factors ($p < 0.05$) obtained from 2^5 factorial designs were further optimized by RSM.

2.9. Central composite design (CCD) and RSM

CCD was employed to determine the optimum level of the significant variables on fibrinolytic enzyme production. The factors used were moisture, pH, and trehalose for enhanced fibrinolytic enzyme production. Each factor in the design was assessed at five levels ($-\alpha$, −1, 0, +1, $+\alpha$) in a set of 20 experiments, including eight factorial, six axial, and six center points as shown in Table 3a. All variables were taken at a central code value that was defined as zero. The fermentation medium was prepared as shown in Table 3b and incubated in the flasks at 37 °C for 72 h. All experiments were conducted in duplicates, and the mean values of fibrinolytic activity (U/mL) were taken as the response (Y). The second-order polynomial equation was employed to fit the experimental data. For a three-factor system, the second-order polynomial equation is as follows (2):

$$Y = \beta_0 + \sum_{i=1}^{3} \beta_0 X_i + \sum_{i=1}^{3} \beta_{ii} X_i^2 + \sum_{ij=1}^{3} \beta_{ij} X_{ij} \quad (2)$$

where Y is the response; β_0 is the offset term; and β_i, β_{ii}, and β_{ij} were the coefficients of linear terms, square terms, and coefficients of interactive terms, respectively. X_is were A, B, and C; X_{ij}s were AB, AC, and BC (A = moisture; B = pH; C = trehalose).

The data obtained from the RSM on fibrinolytic enzyme production were subjected to ANOVA (Table 4). 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 value. The fitted polynomial equation was then expressed as three-dimensional (3D) surface plots to illustrate the relationship between the response and the experimental levels of each of the variables. The statistical software (Design-Expert 8.0.7.0, Stat-Ease Inc.) was used to plot the 3D graphs. The combination of different optimized parameters, which gave maximum response, i.e., maximum fibrinolytic activity was tested experimentally to confirm the validity of the model.

Table 1a
Variables and their levels for 2^5 factorial design for the production of fibrinolytic enzymes.

Symbol	Variable name	Units	Coded levels	
			−1	+1
A	pH		7	9
B	Moisture	%	80	120
C	Trehalose	%	0.25	1
D	Casein	%	0.15	0.75
E	NaH_2PO_4	%	0.05	0.25

2.10. Purification of fibrinolytic enzyme

The crude enzyme was precipitated with ammonium sulphate (70% saturation) and dialyzed against 50 mM Tris–HCl buffer (pH 7.4) containing 10 mM $CaCl_2$ (buffer A) and was loaded on sephadex G-75 gel filtration chromatography. The protein was eluted with the same buffer and the extinction was measured at 280 nm. The active fractions were combined and loaded on casein–agarose affinity column chromatography. This column was equilibrated with buffer A and the bounded proteins were eluted with buffer A containing 50–750 mM NaCl. The extinction was measured at 280 nm and all fractions were subjected to enzyme assay. The casein–agarose affinity chromatography purified fibrinolytic enzyme fractions were subjected to determine the molecular weight of fibrinolytic enzyme. The active fractions obtained from affinity chromatography were used for characterization studies.

2.11. Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) and zymography

Molecular weight analysis of fibrinolytic enzyme was performed by SDS–PAGE

(12%). For SDS–PAGE, enzyme samples were loaded onto gels after being boiled for 1 min in SDS sample buffer. The molecular weight of the enzyme was evaluated with phosphorylase b (97.4 kDa), Bovine Serum Albumin (66 kDa), ovalbumin (43 kDa), carbonic anhydrase (29 kDa), soyabean trypsin inhibitor (20.1 kDa) and lysozyme (14.3 kDa) molecular markers. This gel was silver stained and visualized the protein band. A polyacrylamide gel containing fibrin was prepared by adding 0.12% (w/v) fibrinogen and thrombin (100 NIH U/mL) with the gel (12%). After electrophoresis, the gel was incubated with buffer A containing 2.5% (v/v) Triton X-100 for 30 min at room temperature (30 ± 1 °C). The gel was then washed with double distilled water for 30 min to remove Triton X-100 and incubated with buffer A for 4 h. Finally, the gel was stained with coomassie brilliant blue R-250 for 2 h, after which destained. The bands with fibrinolytic activities were visualized as the nonstained regions on the gel.

2.12. Biochemical properties of purified enzyme

The optimum pH for the activity of the fibrinolytic enzyme was determined using the following buffers (100 mM): citrate buffer (pH 3.0–4.0), succinate buffer (pH 5.0), sodium phosphate buffer (pH 6.0 and 7.0), Tris–HCl buffer (pH 8.0), and glycine–NaOH buffer (pH 9.0 and 10.0). The stability of fibrinolytic enzyme was evaluated by incubating 50 μL enzyme with 50 μL of the above buffers at 37 °C for 1 h and enzyme assay was carried out with substrate. The effect of temperature was determined by assaying

Table 1b
Experimental design and results of 2^5 factorial design of *Shewanella* sp. fibrinolytic enzymes.

Run	pH (A)	Moisture (B)	Trehalose (C)	Casein (D)	NaH_2PO_4 (E)	Enzyme activity (U/mL)
1	1	1	−1	−1	−1	887
2	−1	−1	1	−1	1	338
3	1	−1	−1	−1	1	823
4	−1	1	−1	−1	1	462
5	1	−1	−1	1	1	0
6	1	1	1	1	−1	2136
7	−1	−1	1	1	−1	823
8	−1	1	1	−1	−1	668
9	−1	−1	1	1	1	0
10	1	−1	1	−1	1	1693
11	−1	1	−1	1	1	0
12	1	−1	−1	−1	−1	668
13	1	1	1	−1	−1	1423
14	−1	1	1	−1	1	228
15	−1	1	−1	−1	−1	494
16	1	−1	−1	1	−1	517
17	1	−1	−1	1	1	393
18	1	−1	1	−1	−1	343
19	−1	1	−1	1	−1	594
20	1	1	1	1	1	594
21	−1	−1	−1	1	−1	269
22	1	−1	1	1	1	375
23	−1	−1	−1	−1	−1	727
24	1	1	−1	1	−1	55
25	1	−1	1	1	−1	0
26	−1	1	1	1	1	507
27	−1	−1	−1	−1	1	503
28	−1	1	1	1	−1	462
29	−1	−1	1	−1	−1	0
30	−1	−1	−1	1	1	334
31	1	1	1	−1	1	1478
32	1	1	−1	1	1	1015

the reactions at various temperatures (30–70 °C). To determine the thermal stability, the enzyme was incubated (without substrate) at increasing temperatures (30–70 °C) for 1 h. The effect of ions on enzyme activity was evaluated. The enzyme was incubated with 10 mM of Ca^{2+}, Co^{2+}, Cu^{2+}, Mg^{2+}, Mn^{2+}, Hg^{2+}, Fe^{2+}, Na^{+} and Zn^{2+} ions for 1 h. The enzyme activity was determined as described earlier and the relative enzyme activity was calculated. The plasminogen-free and plasminogen-rich plates were prepared as suggested by Peng et al. [31] to evaluate the efficacy of fibrinolytic enzyme to activate plasminogen and the direct fibrin clot lysis. The prepared fibrin plate was heated at 80 °C for 30 min to inactivate plasminogen present with the commercially available fibrinogen (plasminogen-free plate). Fifteen microliters of fibrinolytic enzyme was placed on the plasminogen-free and plasminogen-rich plates, incubated at room temperature for 4 h.

3. Results and discussion

3.1. Screening of a fibrinolytic enzyme-producing Shewanella sp.

The bacterium *Shewanella* sp. IND20 displayed more protease activity on skimmed milk agar plates and fibrin plates than other bacteria. It produced approximately an 9-mm zone on the fibrin plate, which was higher than the other isolates. The fibrinolytic enzyme production by *Shewanella* sp. IND20 was higher than the other isolates in submerged fermentation. The isolated strain was Gram-positive; rod-shaped; catalase-, citrate-, and oxidase-positive; and had tested negative for indole formation and hydrolysis of urea. It was not able to hydrolyse gelatin and positive to casein-, starch hydrolysis. The 764 bp 16S rDNA sequences of strain were submitted to GenBank database under an accession number KF688984. The 16S rDNA sequences of the strains sharing more than 97% 16S rDNA gene sequence similarity are classified under the same species. The 16S rDNA—based BLAST analysis demonstrate 99% sequence similarity of strain IND20 with other species of the genus *Shewanella*, which suggested that the strain IND20 under study belongs the genus *Shewanella*. The *Shewanella seohaensis* strain S7-3 (NR 108.852.1) and *Shewanella hafnienis* strain P010 (NR 41.296.1) showed 99% 16S rDNA sequence similarity. The strains, *Shewanella putrefaciens* strain Hammer 95 (NR 44.863.1) and *Shewanella putrefaciens* strain NBRC 3908 (NR 113.582.1) showed 98% sequence similarity.

Table 2
ANOVA results for the evaluation of significant factors by 2^5 factorial design.

Source	Sum of squares	df	Mean square	*F* value	*p*-value
Model	7.736E + 006	24	3.223E + 005	33.75	<0.0001
A-pH	1.329E + 006	1	1.329E + 006	139.14	<0.0001
B-Moisture	6.365E + 005	1	6.365E + 005	66.64	<0.0001
C-Trehalose	2.445E + 005	1	2.445E + 005	25.6	0.0015
D-Casein	2.659E + 005	1	2.659E + 005	27.84	0.0012
E-NaH_2PO_4	1.073E + 005	1	1.073E + 005	11.23	0.0122
Residual	66.855.47	7	9550.78		
Cor total	7.802E + 006	31			

Table 3a
Variables and their levels of CCD for the production of fibrinolytic enzymes.

Variables	Symbol	Coded values				
		$-\alpha$	−1	0	+1	$+\alpha$
Moisture (%)	*A*	76.36	90	110	130	143.64
pH	*B*	7.32	8	9	10	10.68
Trehalose (%)	*C*	0.16	0.5	1	1.5	1.83

Table 3b
Experimental design and results of CCD for *Shewanella* sp. fibrinolytic enzymes production.

Run	Moisture (A)	pH (B)	Trehalose (C)	Enzyme activity (U/mL)
1	1(130)	1(10.0)	1(1.5)	2589
2	0(110)	0(9.0)	0(1.0)	1675
3	1(130)	1(10.0)	−1(0.5)	2050
4	0(110)	0(9.0)	0(1.0)	1546
5	0(110)	0(9.0)	−1.682(0.16)	647
6	0(110)	1.682(10.68)	0(1.0)	2296
7	−1(90)	1(10.0)	1(1.5)	1410
8	−1(90)	−1(8.0)	−1(0.5)	1200
9	0(110)	0(9.0)	0(1.0)	1618
10	1(130)	−1(8.0)	1(1.5)	613
11	−1.682(76.36)	0(9.0)	0(1.0)	1010
12	0(110)	0(9.0)	0(1.0)	1600
13	0(110)	0(9.0)	0(1.0)	1500
14	0(110)	−1.682(7.32)	0(1.0)	1011
15	0(110)	0(9.0)	0(1.0)	1674
16	0(110)	0(9.0)	1.682(1.84)	1084
17	1.682(143.64)	0(9.0)	0(1.0)	1380
18	−1(90)	1(10.0)	−1(0.5)	480
19	−1(90)	−1(8.0)	1(1.5)	1521
20	1(130)	−1(8.0)	−1(0.5)	500

Table 4
ANOVA results for the production of fibrinolytic enzymes by CCD and RSM.

Source	Sum of Squares	df	Mean Square	*F* value	*p*-value
Model	6.094E+006	9	6.772E+005	69.4	<0.0001
A-Moisture	2.277E+005	1	2.277E+005	23.33	0.0007
B-pH	1.727E+005	1	1.727E+005	176.98	<0.0001
C-Trehalose	5.095E+005	1	5.095E+005	52.22	<0.0001
AB	2.373E+006	1	2.373E+006	243.21	<0.0001
AC	44850.13	1	44850.13	4.6	0.0576
BC	1.339E+005	1	1.339E+005	13.72	0.0041
A^2	2.331E+005	1	2.331E+005	23.89	0.0006
B^2	17571.4	1	17571.4	1.8	0.2093
C^2	8.558E+005	1	8.558E+005	87.71	<0.0001
Residual	97568.97	10	9756.9		
Lack of fit	73256.14	5	14651.23	3.01	0.1257
Pure error	24312.83	5	4862.57		
Cor Total	6.192E+006	19			

3.2. Agro-residues: an ideal substrates for the production of fibrinolytic enzymes

The marine isolate, *Shewanella* sp. IND20 utilized all agro-residues for its growth and production of fibrinolytic enzymes. Cow dung supported more enzyme production (453 U/mL) than banana peel (48 U/mL), green gram husk (312 U/mL), rice bran (170 U/mL) and wheat bran (221 U/mL) (Fig. 1). Cow dung substrate is available throughout the year than other substrates. The agro-wastes such as *Ficus nitida* waste [14], shrimp shell [36], cotton seed cake [6] and corn cob [8] were reported as the substrate for the production of proteolytic enzymes. The substrates such as soybean grits [36], chickpeas [37], wheat bran [35], spent brewery yeast sludge [38] and soybean meal [39] have been already utilized as potential substrates for fibrinolytic enzyme production. Among all substrates, cow dung is a cheap substrate. The selection of an ideal substrate for enzyme production depends upon the cost and availability of the substrate material [30]. Based on the cheap cost and availability, cow dung is an ideal substrate for the production of fibrinolytic enzymes. Recently, many *Shewanella* sp. have been used as excellent sources for novel industrially useful enzymes [12,27]. Hence, the growth of this organism on cow dung is critically important for the production of industrially useful enzymes in cheap cost.

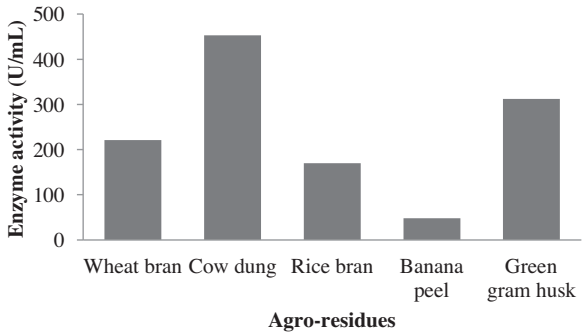

Fig. 1. Evaluation of agro-residues for the production of fibrinolytic enzymes.

3.3. Screening of medium components by one-variable-at-a-time approach

One-factor-at-a-time experiments revealed an increased enzyme production upto 72 h of the fermentation period (1084 U/mL) and decreased after 96 h at 37 °C (942 U/mL). The decreased enzyme production was due to the depletion of nutrients of the fermenting medium. Fibrinolytic enzyme production was high at 100% moisture level (v/w). One-variable-at-a-time results revealed that the enzyme production was found to be maximum at pH 9.5 and decreased on either side of this pH value. Enzyme production was high at a range of 9–12% inoculum (v/w). In an enzyme bioprocess, moisture content of the substrate and pH of the fermentation medium are critically important for enzyme production. In this study, among the non-nutritional factors, pH and moisture of the medium significantly affected fibrinolytic enzyme production. Hence, among non-nutritional factors, pH and moisture were selected for the 2^5 factorial design, using RSM. Fibrinolytic enzyme production was high in the presence of trehalose, and 1884 U/mL of fibrinolytic activity was observed, while relatively less activity (1759, 1101, 1328, and 1431 U/mL, respectively) was observed with other carbon sources (sucrose, starch, glucose, and xylose). Among the nitrogen sources, higher fibrinolytic enzyme activity was obtained with casein (1724 U/mL) when compared with yeast extract, peptone, beef extract, gelatin, and urea (1665, 1555, 1698, 1148, and 827 U/mL, respectively). Among inorganic salts, a higher level of enzyme production was obtained with sodium dihydrogen phosphate (1434 U/mL) when compared with ammonium sulphate, ferrous sulphate, ammonium chloride, disodium hydrogen phosphate, sodium nitrate, and calcium chloride (917, 813, 487, 1027, 714, and 1232 U/mL, respectively). Among the nutrient sources, trehalose supported maximum enzyme prodction (1759 U/mL). One-factor-at-a-time experiments revealed that the nutrient factors such as trehalose, casein, and sodium dihydrogen phosphate significantly affected the fibrinolytic enzyme production. Among all factors, the most significant factors (moisture, pH, trehalose, casein, and sodium dihydrogen phosphate) were selected for statistical optimization.

3.4. Evaluation of medium components affecting fibrinolytic enzyme production using the 2^5 factorial design

The 2^5 factorial experimental design proved to be a valuable tool for the evaluation of the main effects. The results of the 2^5 factorial design have been shown in Table 1b. The fibrinolytic enzyme production in 2^5 factorial design varied between 228 and 2136 U/mL. ANOVA was performed to verify the validity of the models, and the results have been described in Table 2. Based on ANOVA, the "*F*-value" (33.75) for the overall regression model is significant at the 5% level. There is only a 0.01% chance that a "Model *F*-value" this large could occur due to noise. In this model, *A*, *B*, *C*, *D*, *E*, *AB*, *AC*, *AD*,

AE, *BC*, *BD*, *BE*, *DE*, *ABC*, *ABD*, *ABE*, *ACD*, *ADE*, *BCE*, *BDE*, *CDE*, *ABCE*, *ACDE*, and *ABCDE* were significant model terms. The medium pH, moisture, and trehalose positively correlated on fibrinolytic enzyme production, and the increased concentrations of these factors increased enzyme production. These results were in accordance with reported alkaline protease production by microbial strains where enzyme production depends on extracellular pH [32] and in the presence of various sugars [11]. The trehalose is known to effectively stabilize protein [29]. Recently, Nirmal and Laxman [28] studied the effect of trehalose on the stability of fungal protease. The existence of trehalose in the medium is dual importance. First, it stimulates the production of fibrinolytic enzyme and secondly, it protects enzyme from denaturation due to incubation temperature. Addition of casein and NaH_2PO_4 to the medium negatively correlated and the reduction of both concentrations could improve fibrinolytic enzyme production. Enzyme production was enhanced by increasing pH, moisture content, and trehalose concentration significantly ($p < 0.05$) (Table 2). Among the several factors that are important for microbial growth and enzyme production under SSF, moisture content is a critical factor [30]. In the present study there was a significant increase in fibrinolytic enzyme production with an increase in moisture content. The *R* square of this model was 0.991. The predicted *R* square of 0.820 is in reasonable agreement with the adjusted *R*-square of 0.962. The equation in terms of the coded factors is given below:

$$\begin{aligned}\text{Enzyme activity} = {} & +604.34 + 203.78A + 141.03B + 87.41C \\ & - 91.16D - 57.91E + 114.72AB + 109.72AC \\ & - 64.22AD + 46.16AE + 104.22BC \\ & + 82.41BD - 102.22BE - 102.16DE \\ & + 42.53ABC + 91.59ABD - 79.28ABE \\ & - 85.16ACD - 42.84ADE - 92.28BCE \\ & + 55.28BDE - 100.84CDE - 127.09ABCE \\ & - 75.66ACDE - 147.34ABCDE. \end{aligned} \quad (3)$$

On the basis of calculated *t*-values, the moisture content, pH, and trehalose were selected for further optimization using CCD and RSM.

3.5. Response surface methodology

RSM is a powerful technique for testing multiple process variables, because fewer experimental trials are required when compared with the study of one-variable-at-a-time experiment. Also, interactions between variables can be identified and quantified by this technique [9]. The optimum concentration of the most significant factors (moisture, pH, and trehalose) was investigated further using CCD and RSM. The CCD model helps to study the interactions between the various variables, and RSM helps to explore the optimum concentrations of each of the variables. RSM had been successfully used for the enhancement of fibrinolytic enzyme production by many bacterial species [4,10].

The second-order polynomial model was used to correct the independent variables with fibrinolytic activity. The highest activity of the fibrinolytic protease that was observed was at 2589 U/mL at run 1 (Table 3b). The model *F*-value of 69.40 implied that the model was significant. There is only a 0.01% chance that a "Mode *F*-value" of this magnitude could occur due to noise. The data obtained were best fitted into a quadratic model. The regression analysis of the experimental design showed that the linear model terms (*A*, *B*, and *C*), interactive model terms (*AB*, *AC*, and *BC*), and the quadratic model terms (A^2 and C^2) were significant. However, the quadratic model term B^2 was found to be insignificant ($p > 0.05$). The value of the multiple correlation coefficient (R^2) was found to be 0.97 (a value >0.75 indicates aptness of the model), which indicated that the model can explain 97% of the variation in the response. The adjusted coefficient (R^2) obtained for the model was 0.805. According to ANOVA, the lack of fit is insignificant, indicating that the second-order model with

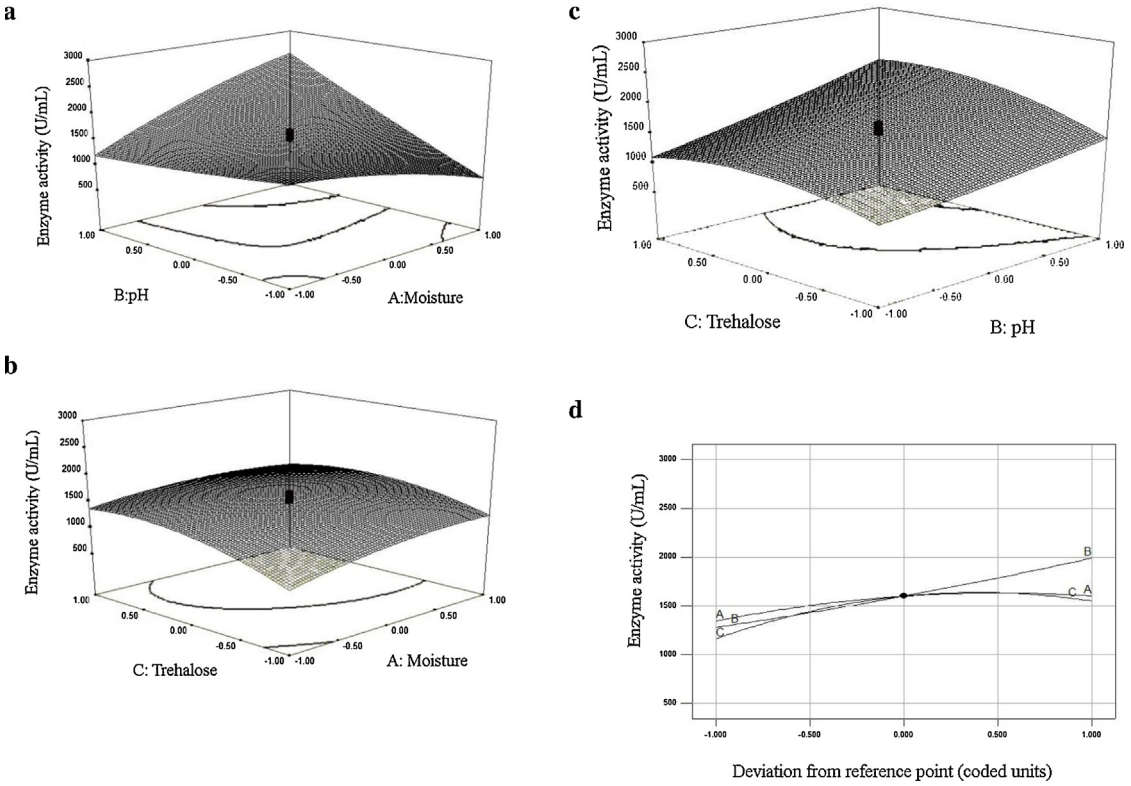

Fig. 2. Response surface plots showing the effect of pH and moisture (a), trehalose and moisture (b), and trehalose and pH (c), perturbation graph shows the effect of moisture, pH and trehalose (d).

interaction is very adequate in approximating the response surface of the experimental design (Table 4). The lack-of-fit *F*-value of 3.01 implies that there is a 12.57% chance that a large lack-of-fit *F*-value could occur due to noise. The model showed coefficient of variation (CV), standard deviation, mean, and predicted residual sum of square values of 24.64, 350.31, 1421.80, and 6.185E + 006, respectively. Applying multiple regression analysis, the results were fitted in to a second-order polynomial equation (2).

The 3D response surface curves were plotted to determine the optimum concentration of each factor for maximum fibrinolytic enzyme production (Fig. 2a–c). The results clearly showed a strong degree of curvature of 3D surface, from where the optimum concentration was determined. The fibrinolytic enzyme production varied significantly upon changing the initial moisture content and pH of the medium. These environmental factors have been found to be affecting the production of fibrinolytic enzymes. The 3D plots depicted that there was an increased enzyme production up to pH 10 and then depleted thereafter. Alkaliphiles are defined as organisms that grow optimally at alkaline pH, with pH optima for growth being in excess of pH 8.0 [16]. The marine isolate *Shewanella* sp. IND20, produced significant amount of fibrinolytic enzyme at alkaline pH (10), and this organism is called as alkaliphiles. The increasing concentrations of trehalose increased fibrinolytic enzyme production. A low value (7.21%) of the coefficient of variance indicates a high degree of precision and good reliability of experimental values. The RSM clearly showed that pH of the medium greatly affected the production of the fibrinolytic enzyme when compared with other factors. To understand the effect of all the three factors, a perturbation plot was generated from this experiment (Fig. 2d). It clearly demonstrated the significant increase of enzyme production at higher pH values than other factors. The "adequate precision value" is an index of the measure of signal (controllable factors) to noise (uncontrollable), and a value greater than four is prerequisite for a model to be a good fit. In this model, a ratio of 29.481 indicates an adequate signal, and this model can be used to navigate the design space. The regression equation coefficient was calculated, and the data were fitted in to a second-order polynomial equation as given below:

$$\begin{aligned}\text{Fibrinolytic activity}(Y) = {} & +1599.60 + 129.11A + 355.58B \\ & + 193.16C + 544.63AB - 74.87AC \\ & + 129.38BC - 127.19A^2 + 34.92B^2 \\ & - 243.68C^2 \end{aligned} \quad (4)$$

where *A* is moisture, *B* is beef extract, and *C* is NaH_2PO_4.

The resulting optimum medium composition was 120.81% moisture, 1.21% trehalose, at pH 10. The predicted fibrinolytic enzyme activity was estimated at 2618 U/mL. In order to confirm the predicted result, experiments were performed in triplicates using the optimized conditions and a fibrinolytic activity of 2751 U/mL was obtained. This experimental value was in good agreement with that of predicted value validated the model. The optimized medium showed 2.5-fold increase on enzyme production than unoptimized medium. This 2.5-fold increase was comparatively higher than the earlier report of RSM on *B. subtilis* [10] and *Bacillus* sp. strain AS-S20-1 [26]. Response surface methodology is the powerful tool for optimized production of enzymes [10,15,26].

3.6. SDS-PAGE and fibrin zymography

The fibrinolytic enzyme was purified to electrophoretic homogeneity after casein–agarose affinity chromatography purification. The specific activity of the fibrinolytic enzyme increased to 334 units/mg protein with a 47.9% yield after gel filtration chromatography. After the elution of the fibrinolytic enzyme by casein–agarose affinity chromatography, the specific activity of the protein increased to 573 units/mg protein with 17% yield. The apparent molecular weight of the purified fibrinolytic enzyme was estimated to be approximately 55.5 kDa (Fig. 3a). The molecular weight of alkaline serine protease of *Shewanella* strain Ac10 was reported as 85 kDa [20] and the fibrinolytic enzyme from *Shewanella* sp. was not reported.

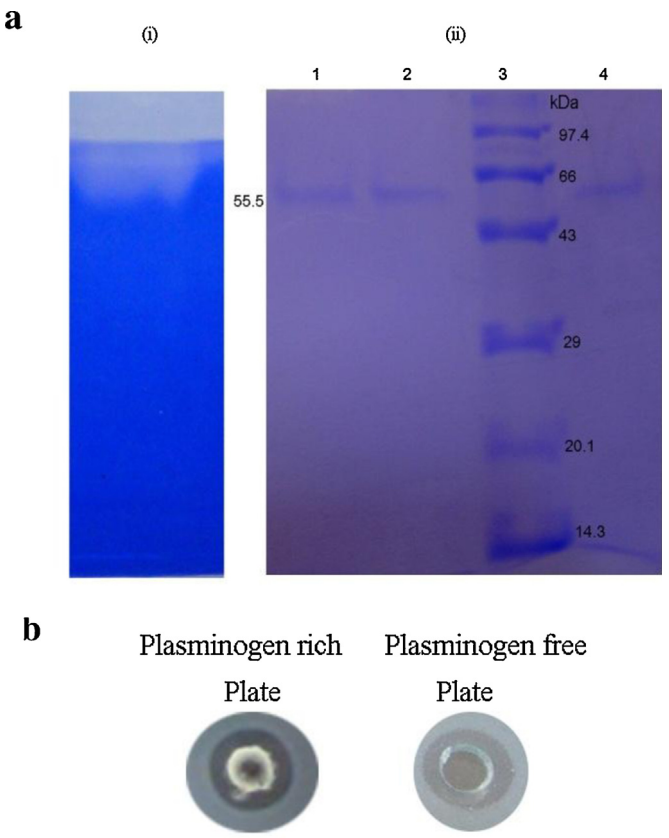

Fig. 3. (a) Sodium dodecyl sulphate polyacrylamide gel electrophoresis (12%) of the purified fibrinolytic enzyme from *Shewanella* sp. (i): fibrinolytic activity appeared as colourless band after stained with CBB, (ii): lane 1, 2 and 4-purified enzyme; lane 3—relative molecular mass standards). (b) Activity of *Shewanella* sp. fibrinolytic enzyme on plasminogen-rich and plasminogen-free plates.

3.7. Biochemical properties of fibrinolytic enzyme

This fibrinolytic enzyme was active at neutral and alkaline pH values, and optimal reaction for fibrinolytic enzyme was obtained at pH 8.0. This optimum pH was similar to that of *B. subtilis* HQS-3, and *Streptomyces* sp. fibrinolytic enzyme [18,40]. The enzyme was stable at pH 8.0 and decreased considerably at higher pHs. At varying temperatures, this enzyme exhibited maximal activity at 50 °C, and was stable upto 40 °C after 1 h incubation. It was reported that the nattokinase stability was less than 50 °C [41] and the present study fell this range. The effect of metal ions on the fibrinolytic activity was also examined. Ca^{2+} and Mg^{2+} activated the fibrinolytic activity and the relative enzyme activity was 108%, and 128%, respectively. Other than these two ions, none of the ions activated the fibrinolytic activity. The enzyme secreted by *Shewanella* sp. IND20 showed direct fibrin clot lysis activity and PA activity on plasminogen-free and plasminogen-rich plates. Enzyme activity was found to be high in plasminogen-rich plate than the plasminogen-free plate (Fig. 3b). The fibrinolytic enzyme could lyse clot *in vitro* effectively, suggesting that it could be used as an effective thrombolytic agent. Hua et al. [17] have reported that the fibrinolytic enzyme from *Bacillus* sp. nov. SK006 degraded

fibrin clot in plasminogen-rich plate by forming active plasmin from the plasminogen and in plasminogen-free plate by direct fibrinolysis, and the activity was more in plasminogen-rich plate. The degradation of fibrin on both plasminogen-rich and plasminogen-free plate suggested predominant production of fibrinolytic enzyme from *Shewanella* sp. IND20 may have great applications in the treatment of CVDs.

4. Conclusions

The newly isolated *Shewanella* sp. IND20 utilized cow dung for the production of fibrinolytic enzyme. This cow dung substrate may have wide application in enzyme industry in future. This enzyme effectively degrade fibrin clot directly and activated plasminogen to form active plasmin. This study explores new source of fibrinolytic enzyme from *Shewanella* sp. IND20 to treat and prevent CVDs.

Acknowledgement

The author P. Vijayaraghavan gratefully acknowledges the Council for Scientific and Industrial Research (CSIR), India for providing a Senior Research Fellowship (Ref: 09/652(0024)/2012 EMR-1).

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