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Electronic Journal of Biotechnology 20 (2016) 1–8

Contents lists available at ScienceDirect

Electronic Journal of Biotechnology

Research Article

# Bioconversion of agro-industrial wastes for the production of fibrinolytic enzyme from *Bacillus halodurans* IND18: Purification and biochemical characterization

Ponnuswamy Vijayaraghavan [a,*], Samuel Gnana Prakash Vincent [a], Mariadhas Valan Arasu [b], Naif Abdullah Al-Dhabi [b]

[a] *International Centre for Nanobiotechnology, Centre for Marine Science and Technology, Manonmaniam Sundaranar University, Rajakkamangalam 629 502, Kanyakumari District, Tamil Nadu, India*
[b] *Department of Botany and Microbiology, Addiriyah Chair for Environmental Studies, College of Science, King Saud University, P.O. Box 2455, Riyadh 11451, Saudi Arabia*

ARTICLE INFO

*Article history:*
Received 13 October 2015
Accepted 14 January 2016
Available online 27 January 2016

*Keywords:*
Agro-residues
Solid-state fermentation
Statistical approach
Thrombolytic agent

ABSTRACT

*Background:* Agro-wastes were used for the production of fibrinolytic enzyme in solid-state fermentation. The process parameters were optimized to enhance the production of fibrinolytic enzyme from *Bacillus halodurans* IND18 by statistical approach. The fibrinolytic enzyme was purified, and the properties were studied.
*Results:* A two-level full factorial design was used to screen the significant factors. The factors such as moisture, pH, and peptone were significantly affected enzyme production and these three factors were selected for further optimization using central composite design. The optimum medium for fibrinolytic enzyme production was wheat bran medium containing 1% peptone and 80% moisture with pH 8.32. Under these optimized conditions, the production of fibrinolytic enzyme was found to be 6851 U/g. The fibrinolytic enzyme was purified by 3.6-fold with 1275 U/mg specific activity. The molecular mass of fibrinolytic enzyme was determined by sodium dodecyl sulphate polyacrylamide gel electrophoresis, and it was observed as 29 kDa. The fibrinolytic enzyme depicted an optimal pH of 9.0 and was stable at a range of pH from 8.0 to 10.0. The optimal temperature was 60°C and was stable up to 50°C. This enzyme activated plasminogen and also degraded the fibrin net of blood clot, which suggested its potential as an effective thrombolytic agent.
*Conclusions:* Wheat bran was found to be an effective substrate for the production of fibrinolytic enzyme. The purified fibrinolytic enzyme degraded fibrin clot. The fibrinolytic enzyme could be useful to make as an effective thrombolytic agent.

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## 1. Introduction

Cardiovascular diseases (CVDs) such as acute myocardial infarction, ischemic heart diseases, and stroke are the leading cause of death worldwide, and the number of deaths due to CVDs is expected to increase over 23.3 million by 2030 [1]. The fibrinolytic agents such as urokinase plasminogen activator (u-PA), tissue plasminogen activator (t-PA), and streptokinase are commonly used to treat CVDs. However, the thrombolytic agents such as t-PA and u-PA are generally safe but are very expensive; later is a cheap fibrinolytic agent but causes undesirable side effects such as gastrointestinal bleeding [2]. Considering the global burden, the search continues for a safe and cheap thrombolytic agent to treat CVDs. In recent years, many studies on bacterial fibrinolytic enzymes have been proven as a safe thrombolytic agent. Likewise, Mine et al. [3] described the use of fibrinolytic enzymes from food-grade microorganisms. The bacterial strains such as *Bacillus subtilis* A26 [4], *Bacillus amyloliquefaciens* [5], *Paenibacillus* sp. IND8 [6], *Xenorhabdus indica* KB-3 [7], and *Bacillus* sp. [8] were used for the production of fibrinolytic enzymes in recent years. Bacterial fibrinolytic enzymes were produced by both submerged fermentation and solid-state fermentation (SSF).

SSF is a potential technology for the production of various biomolecules including enzymes. The cheap and easily available agro-industrial wastes such as apple pomace [9,10], rice chaff [11], pigeon pea [12], potato peel [13], and coffee pulp [14] were used as the sole source of carbon for the production of proteolytic enzymes in SSF. In SSF, the enzyme yield is relatively higher than submerged

* Corresponding author.
*E-mail address:* venzymes@gmail.com (P. Vijayaraghavan).
Peer review under responsibility of Pontificia Universidad Católica de Valparaíso.

http://dx.doi.org/10.1016/j.ejbt.2016.01.002
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fermentation process. The main advantage of SSF is to utilize cheap agro-industrial wastes as the substrate for the production of enzymes. Statistical experimental design has many advantages over traditional one-at-a-time optimization strategy. The statistical tool such as fractional factorial design [15], Plackette-Burman's design [16], and L-18 orthogonal array [17] was frequently used in enzyme bioprocess. One of the important tasks in an enzyme bioprocess is the production of enzyme in cheaper cost. Hence, optimization of process parameters by statistical approach, such as two-level full factorial design and Response Surface Methodology (RSM), could improve the enzyme yield significantly and decreases the production cost of enzymes. The traditional one-at-a-time strategy method of optimization frequently failed to locate the optimum response. RSM has been frequently applied for the production of enzymes such as nattokinase [15], glucose oxidase [18], arginine deiminase [19], L-asparaginase [20], and laccase [21], and so on. The report on statistical optimization of fibrinolytic enzyme production in SSF is limited. The main aim of this study was to optimize the medium components by RSM to enhance fibrinolytic enzyme production in SSF using agro-industrial wastes and to study the properties of enzyme.

## 2. Materials and methods

### *2.1. Screening of fibrinolytic enzyme producing bacterial isolate*

Rice was boiled in water for 45 min and was allowed for aerobic fermentation for 48 h at room temperature (30 ± 2°C). This fermented rice was used as the source of fibrinolytic enzyme producing bacteria. Ten grams of fermented rice was suspended in double distilled water and screened on skimmed milk agar plates for isolation of proteolytic bacteria. Thirteen bacterial isolates showed halo zone on skimmed milk agar plates were further cultured using wheat bran substrate in SSF. The pH of the substrate was adjusted as 9.0 using glycine-NaOH buffer (0.1 M), and 80% (v/w) moisture level were maintained. The substrate was autoclaved at 121°C for 15 min, and SSF was initiated by inoculating individual bacterial isolates (10%, v/w). These flasks were incubated at 37°C for 72 h, after which 50 mL double distilled water was added and placed in an orbital shaker for 30 min at 175 rpm. It was further centrifuged at 5000 × g for 20 min at 4°C, and the supernatant was used as the source of crude enzymes. Fibrinolytic activity of the crude enzyme of the bacterial isolates was tested individually using a fibrin plate composed of 1% (w/v) agarose, 0.5% (w/v) fibrinogen, and 50 μL (100 NIH U/mL) thrombin [22]. This plate was allowed to stand for 1 h at room temperature (30°C ± 2°C) to form a fibrin clot layer, and 10 μL of crude enzyme from the individual isolate was dropped into wells. The plates were incubated at 37°C for 5 h, and the fibrinolytic activity exhibited a clear zone of fibrin degradation around the well.

### *2.2. Identification of the bacterial isolate*

In the present study, 10 bacterial isolates showed fibrinolytic activity, and the potent bacterial isolate was used for further studies. The potent bacterial isolate was identified using its morphological, biochemical characters [23] and 16S rRNA sequencing using forward (P1: 5′-AGAGTTTGATCMTGGCTAG-3′) and reverse primer (P2: 5′-ACGGGCGGTGTGTRC-3′). The DNA was amplified by using a Peltier Thermal Cycler Machine (USA) and DNA polymerase (Sigma-Aldrich, USA). The resulted PCR amplicon was sequenced and compared the sequence with the database using BLAST NCBI server [24]. The 898 bp 16S rDNA sequences of the bacterial isolate were submitted to GenBank.

### *2.3. Substrate*

The agro-industrial wastes such as banana peel, black gram husk, cow dung, paddy straw rice bran, and wheat bran were collected from Nagercoil, Kanyakumari district, Tamilnadu, India. These substrates were dried individually under sunlight for a week and powdered using a mixer grinder. Finally, these substrates were stored in a container individually for further use.

### *2.4. Screening of various agro-industrial wastes for the production of fibrinolytic enzymes*

5.0 g of processed substrate (banana peel, black gram husk, cow dung, paddy straw, rice bran, and wheat bran) was taken in a 100-mL Erlenmeyer flask individually. These substrates were moistened with glycine-NaOH buffer (pH 9.0, 0.1 M) at 80% (v/w) level. The contents were mixed thoroughly and autoclaved at 121°C for 20 min. The sterilized medium was inoculated with 18 h grown *Bacillus halodurans* IND18 at 10% (v/w) level and incubated for 72 h at 37°C. The fibrinolytic enzyme was extracted from agro-residues using 50 mL glycine-NaOH buffer (pH 9.0, 0.1 M) by shaking on a rotary shaker at 150 rpm for 30 min. The clear supernatant was used as the crude enzyme.

### *2.5. Assay of fibrinolytic activity*

Fibrinolytic enzyme activity of the culture supernatant was carried out using fibrin substrate [25]. The reaction mixture contained 2.5 mL fibrin (1.2%, w/v), 2.5 mL Tris–HCl buffer (0.1 M, 0.01 M $CaCl_2$, pH 7.8), and 0.1 mL crude enzyme. It was incubated at 37°C for 30 min, and the reaction was stopped by the addition of 5 mL of 0.11 M trichloroacetic acid containing 0.22 M sodium acetate and 0.33 M acetic acid. The reaction mixture was centrifuged for 15 min at 10,000 × g, and the clear supernatant was read at 275 nm using a UV–visible spectrophotometer. One fibrinolytic unit was defined as the amount of enzyme that gave an increase in absorbency at 275 nm equivalent to 1 μg of tyrosine/min at 37°C.

### *2.6. Screening of process parameters by one variable-at-a-time strategy*

In the present study, wheat bran was used as the substrate for optimization of enzyme production in SSF. The process parameters such as fermentation period (24–96 h), pH (6.0–10), inoculum (2–12%), moisture content (40–100%), carbon sources (1% maltose, sucrose, starch, glucose, and trehalose), nitrogen sources (1% yeast extract, peptone, beef extract, gelatine, and urea), and inorganic ions (0.01% ammonium sulphate, ammonium chloride, disodium hydrogen phosphate, sodium nitrate, sodium dihydrogen phosphate, calcium chloride, and dipotassium hydrogen phosphate) on the production of enzymes were studied.

### *2.7. Screening of significant factors by statistical approach*

The medium constituents toward fibrinolytic enzyme production were screened by a full factorial experimental design. The significant physical parameters (pH and moisture) and the nutritional factors (glucose, peptone, and $CaCl_2$) were subjected for screening. The $2^5$ factorial design consisted of 32 experimental runs in which the selected five factors such as pH, moisture, glucose, peptone, and $CaCl_2$ were kept either at their low (-) or high (+) levels. The other process parameters such as fermentation period and inoculum were kept at optimum level. The statistically designed culture media were prepared according to the designed protocol and inoculated with the seed culture at 10% level (v/w) and incubated at 37°C for 72 h. At the end of the fermentation period, fibrinolytic

**Table 1**
The factors and levels (low and high) selected for $2^5$ full factorial design.

| Symbol | Variable name | Units | Coded levels | |
|---|---|---|---|---|
| | | | -1 | +1 |
| A | Moisture | % | 60 | 100 |
| B | pH | | 7 | 9 |
| C | Glucose | % | 0.1 | 1 |
| D | Peptone | % | 0.1 | 1 |
| E | $CaCl_2$ | % | 0.01 | 0.1 |

enzyme assay was carried out. The variables and levels (-1 and +1) were explained in Table 1. All these 32 experimental runs were carried out in a 100-mL Erlenmeyer flask containing 5 g of wheat bran with appropriate physical and nutritional factors. Design-Expert 9.0.7.1 (StatEase Inc., Minneapolis, MN, USA) was used to design and to analyse the experimental results. Two-level full factorial design was based on the following first-order polynomial model:

$$Y = \alpha 0 + \Sigma \alpha ixi + \Sigma \alpha ijxixj + \Sigma \alpha ijkxixjxk + \Sigma \alpha ijklxixjxkxl + \Sigma \alpha ijklmxixjxkxlxmiijijkijklijklm, \quad \text{[Equation 1]}$$

where $Y$ is the response (fibrinolytic activity); $\alpha_{ij}$, $\alpha_{ijk}$, $\alpha_{ijkl}$, and $\alpha_{ijklm}$ were the *ij*th, *ijk*th, *ijkl*th, and *ijklm*th interaction coefficients, respectively; $\alpha_i$ was the *i*th linear coefficient; and $\alpha_0$ an intercept. The statistical parameters were estimated using analysis of variance, and the values of "Prob > *F*" less than 0.05 indicated that the model terms were statistically significant. The significant factors were further selected for optimizing the concentration of factors using central composite design (CCD) and RSM.

### *2.8. CCD and response surface methodology*

CCD was employed to optimize the concentrations of significant factors influencing fibrinolytic enzyme production. The three test variables such as moisture, pH, and peptone were employed to fit a second-order polynomial model using a $2^3$ full factorial design. The CCD composed of a set of 20 experimental runs. For a three-factor system, the second-order polynomial equation is as follows:

$$Y = \sum_{I=1}^{3} \beta 0 + \sum_{i=1}^{3} \beta iXi + \sum_{ij=1}^{3} \beta iiXi2 + \Sigma \beta ijXij \quad \text{[Equation 2]}$$

where $Y$ is the response; $\beta_0$ is the offset term; $\beta_i$, $\beta_{ii}$, and $\beta_{ij}$ are the coefficients of linear terms, square terms, and interactive terms, respectively. $X_i$s are A, B, and C, and $X_{ij}$s are AB, AC, and BC (A is a coded value of moisture, B is a coded value of pH, and C is a coded value of peptone). All these 20 experimental runs were carried out in a 100 mL Erlenmeyer flask containing 5 g of wheat bran with the designed medium. The fibrinolytic enzyme assay was carried out in duplicates, and the average of these experimental results was taken as response *Y*. The optimized concentration of variables, which gave maximum response, was tested experimentally to confirm the validity of the model. The statistical analysis was carried out by Design Expert software package (Version 9, StatEase Inc.).

### *2.9. Purification of fibrinolytic enzyme*

The crude fibrinolytic enzyme was precipitated with ammonium sulphate (70% saturation) and dialyzed over night against double distilled water and buffer A (0.05 M Tris–HCl buffer, 8.0). The dialyzed sample was applied to a Diethylaminoethyl cellulose (DEAE) column chromatography (1.2 × 10 cm) (Merck, India). It was previously equilibrated with buffer A and eluted with buffer A containing 0–1.0 M NaCl at a flow rate of 1.0 mL/min. The fractions containing fibrinolytic activity were pooled and loaded on casein-agarose affinity chromatography (Sigma-Aldrich, USA). The proteins were eluted with buffer A containing 0.1–0.8 M NaCl. The extinction of the eluted fractions was measured at 280 nm, and the enzyme assay was carried out.

### *2.10. Sodium dodecyl sulphate polyacrylamide gel electrophoresis and zymography analysis*

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) analysis was performed using 12% separating and 5% stacking gel [26]. Zymography analysis was carried out by substituting 0.12% fibrinogen and 10 NIH U/mL thrombin with SDS-PAGE [27]. Further, the PAGE-separated gel was washed with buffer A containing 20 mM $CaCl_2$ and incubated the gel overnight at 37°C. Then, the gel was stained with CBB and distained; the fibrinolytic activity appeared as a colourless band with a blue background.

### *2.11. Properties of fibrinolytic enzyme*

The effect of pH on enzyme activity was determined by reacting enzyme solution with substrate at various pHs. To evaluate the optimal pH, fibrinolytic enzyme was assayed at various pHs (3.0–10.0). To check the stability of the enzyme at various pHs, the enzyme was pretreated with buffer (pH 3.0–10.0), and the reaction mixture was further used to measure the fibrinolytic activity. The effect of temperature on enzyme activity was determined by performing the reactions at various temperatures (30°C–70°C). To determine the thermal stability, the sample was preincubated for 1 h at various temperature ranges from 30°C to 70°C without substrate; further enzyme assay was carried out. To study the effect of ions on enzyme activity, the enzyme was incubated with ions such as $Ca^{2+}$, $Co^{2+}$, $Cu^{2+}$, $Mg^{2+}$, $Mn^{2+}$, $Hg^{2+}$, $Fe^{2+}$, $Na^{+}$, and $Zn^{2+}$, and the residual enzyme activity was assayed. The clot lytic property of enzyme was carried out *in vitro*. About 2 mL blood was collected from healthy male volunteer with written informed consents, and blood clot was made by spontaneous coagulation in the centrifuge vials, and fibrinolytic enzyme was added and incubated to study it *in vitro* clot lytic activity. In this study, buffered saline was used as the negative control, and streptokinase was used as the positive control. These vials were incubated at room temperature (30°C ± 2°C) for 60 min, and blood clot lytic activity was analysed. The plasminogen-free and plasminogen-rich plates were prepared to evaluate the efficacy of fibrinolytic enzyme as plasminogen activator activity and direct fibrin clot lysis activity. To prepare plasminogen-free plate, the fibrin plate was subjected at 80°C in a water bath for 30 min to inactivate plasminogen. Further, 10 μg and 20 μg of fibrinolytic enzyme was dropped on the plasminogen-free and plasminogen-rich plates and incubated at room temperature (30 ± 2°C) for 4 h. The fibrinolytic enzyme activity appeared as a clear zone of degradation on these plates. All experiments were carried out in triplicates.

## 3. Results and discussion

### *3.1. Screening of fibrinolytic enzyme-secreting organisms*

In the present study, 10 bacterial strains showed activity on fibrin plate (Fig. 1). Based on fibrinolytic activity, the organism that showed large zones was used for fibrinolytic enzyme production, and the organism was identified as *B. halodurans* IND18. The identified organism was Gram positive; rod shaped; starch hydrolysing; citrate, catalase, oxidase, and nitrate positive. This organism was not able to produce $H_2S$ and hydrolysed casein. This was further identified by 16S rRNA sequencing, and the accession number was assigned (KF 688986).

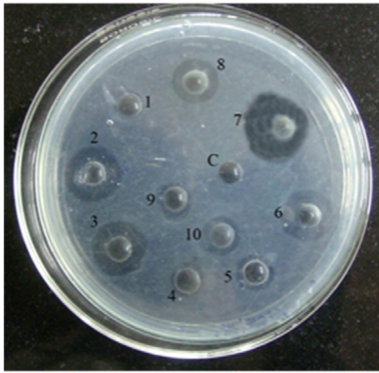


**Fig. 1.** Screening of fibrinolytic enzyme from the selected bacteria. The crude fibrinolytic enzyme was tested individually using a fibrin plate. The fibrinolytic activity exhibited a clear zone of fibrin degradation around the well (1–10: crude extract from 10 bacterial isolates; C: control).

### *3.2. Screening of agro-wastes for fibrinolytic enzyme production in SSF*

The results of the present study proved that fibrinolytic enzyme production was higher in wheat bran substrate than other tested substrates (Fig. 2). Wheat bran had obvious effect on enzyme production (2450 ± 103 U/g) than that of cow dung substrate, which showed 2217 ± 85 U/g substrate. The results showed that varying fibrinolytic enzyme production depends on the type of substrate used as fermentation medium. In SSF, selection of an ideal substrate is a key factor, and it should be available throughout the year, and it should be cheap [14]. Based on the results obtained, the cheap agro residue wheat bran is a substrate of choice for the production of fibrinolytic enzyme for this isolate.

### *3.3. Screening of process parameters by one-variable-at-a-time approach*

The physical and nutrient factors were screened by a traditional one-factor-at-a-time approach. Fibrinolytic enzyme production was found to be high (2651 U/g) after 72 h of fermentation period, and enzyme production was 1027, 1888, and 2107 U/g after 24, 48, and 96 h, respectively. This result on the fermentation period of *B. halodurans* for fibrinolytic enzyme production found in the present study was in accordance with the findings of George-Okafor and Mike-Anosike [28] who stated that the optimum fermentation period was 72 h for *Bacillus* sp. SW-2. Enzyme production was maximum at 80% moisture content (2754 U/g), pH 9.0 (4209 U/g), and 9% inoculum level (2628 U/g). Moisture content is one of the critical factors in enzyme bioprocess [14], and it was previously reported that the optimum moisture content of the fermentation medium varies based on the type of substrate used in fermentation process [29,30]. Among the carbon and nitrogen sources, glucose (2854 U/g) and peptone (3093 U/g) supplemented medium showed more enzyme production. It was reported that glucose is a preferred carbon and energy source for *Bacillus* sp. [31]. Bacteria generally required complex nitrogen sources for protease production, and the requirement of specific nitrogen sources differs from organism to organism [32]. In this study, peptone was found to be a suitable nitrogen source. Mukesh Kumar et al. [33] also stated that peptone was found to be suitable for the production of fibrinolytic enzyme from *B. subtilis* RJAS19. Fibrinolytic enzyme production was considerably high in the presence of calcium as the sole ionic source (2698 U/g); however, other ions did not influence fibrinolytic enzyme production. Recently, Mahajan et al. [17] also observed the positive effect of calcium ion to enhance fibrinolytic enzyme production from *B. subtilis* ICTF-1.

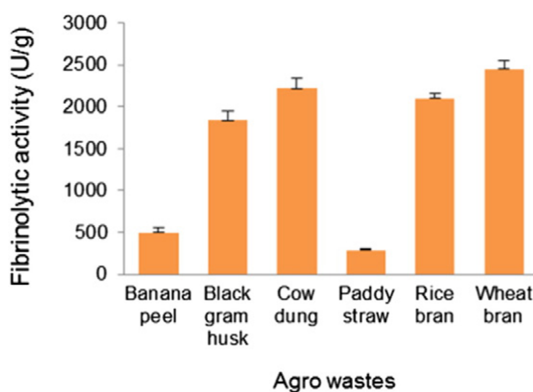


**Fig. 2.** Production of fibrinolytic enzyme by *B. halodurans* IND18 using various agro residues. Five grams of substrate was taken in 100-mL Erlenmeyer flask individually and SSF was carried out.

### *3.4. Screening of significant factors by statistical approach*

The $2^5$ full factorial design model showed that the fibrinolytic enzyme production varied from 13 to 6314 U/g (Table 2). The *F*-value of this model was 10.64, which implied that this model was statistically significant ($p < 0.05$). In this model, the factors such as A, C, AB, AC, BC, $A^2$, and $B^2$ were significant (Table 3). The “adequate precision” ratio of this model was 11.57, which indicated an adequate signal. The model equation for fibrinolytic enzyme production from *B. halodurans* strain IND18 can be written as follows:

**Table 2**
Response of $2^5$ factorial design for fibrinolytic enzyme by *B. halodurans* IND18.

| Run | Moisture (A) | pH (B) | Glucose (C) | Peptone (D) | $CaCl_2$ (E) | Enzyme activity (U/g) |
|---|---|---|---|---|---|---|
| 1 | -1 | 1 | 1 | 1 | 1 | 4896 |
| 2 | -1 | 1 | -1 | -1 | -1 | 4882 |
| 3 | -1 | -1 | -1 | -1 | 1 | 1619 |
| 4 | 1 | -1 | 1 | 1 | 1 | 13 |
| 5 | 1 | -1 | -1 | 1 | 1 | 1015 |
| 6 | -1 | -1 | -1 | 1 | -1 | 3809 |
| 7 | 1 | -1 | 1 | -1 | 1 | 2031 |
| 8 | -1 | -1 | 1 | -1 | -1 | 6213 |
| 9 | 1 | 1 | -1 | 1 | 1 | 5568 |
| 10 | -1 | -1 | 1 | 1 | 1 | 4397 |
| 11 | 1 | -1 | 1 | -1 | -1 | 3697 |
| 12 | -1 | 1 | 1 | -1 | 1 | 3688 |
| 13 | 1 | 1 | -1 | -1 | 1 | 3029 |
| 14 | 1 | 1 | 1 | -1 | -1 | 4438 |
| 15 | 1 | 1 | 1 | 1 | 1 | 1995 |
| 16 | 1 | 1 | -1 | -1 | -1 | 5134 |
| 17 | 1 | 1 | 1 | -1 | 1 | 1102 |
| 18 | 1 | -1 | -1 | 1 | -1 | 183 |
| 19 | -1 | -1 | 1 | 1 | -1 | 1130 |
| 20 | 1 | 1 | -1 | 1 | -1 | 1015 |
| 21 | 1 | -1 | -1 | -1 | -1 | 3366 |
| 22 | -1 | -1 | -1 | -1 | -1 | 5500 |
| 23 | 1 | 1 | 1 | 1 | -1 | 2873 |
| 24 | 1 | -1 | -1 | -1 | 1 | 3834 |
| 25 | -1 | -1 | -1 | 1 | 1 | 5509 |
| 26 | -1 | 1 | 1 | -1 | -1 | 5422 |
| 27 | -1 | 1 | -1 | -1 | 1 | 4850 |
| 28 | -1 | 1 | -1 | 1 | -1 | 4621 |
| 29 | 1 | -1 | 1 | 1 | -1 | 2544 |
| 30 | -1 | -1 | 1 | -1 | 1 | 5587 |
| 31 | -1 | 1 | -1 | 1 | 1 | 6314 |
| 32 | -1 | 1 | 1 | 1 | -1 | 5056 |

**Table 3**
ANOVA for $2^5$ factorial experimental design for the production of fibrinolytic enzyme by *B. halodurans* IND18.

| Source | Sum of squares | df | Mean square | *F* value | *p*-Value |
|---|---|---|---|---|---|
| Model | 1.052E+008 | 28 | 3.756E+006 | 10.64 | 0.0373 |
| A—Moisture | 3.053E+007 | 1 | 3.053E+007 | 86.46 | 0.0026 |
| B—pH | 5.981E+006 | 1 | 5.981E+006 | 16.94 | 0.026 |
| C—Glucose | 9.678E+005 | 1 | 9.678E+005 | 2.74 | 0.1964 |
| D—Peptone | 6.173E+006 | 1 | 6.173E+006 | 17.48 | 0.0249 |
| E—$CaCl_2$ | 7.929E+005 | 1 | 7.929E+005 | 2.35 | 0.231 |
| Residual | 1.059E+006 | 3 | 3.531E+005 | | |
| Cor total | 1.062E+008 | 31 | | | |

Final equation in terms of coded factor:

$$\begin{aligned}\text{Enzyme activity} &= +3622.84-976.78A+432.34B-173.91C-439.22D-157.41E \\ &+ 65.84AB-135.53AC-306.09AD-165.28AE-197.53BC+436.28BD \\ &+ 32.47BE-146.72CD-327.91CE+687.16DE-35.28ABC+37.53ABD \\ &+ 69.47ABE+411.66ACD-400.78ACE-117.47ADE \\ &+ 180.91BCD-310.66BCE+88.78BDE-239.59CDE-332.34ABCD \\ &+ 206.59ABCE+481.03ABDE \end{aligned} \quad \text{[Equation 3]}$$

where A is moisture, B is pH, C is glucose, D is peptone, and E is $CaCl_2$.

In this model, enzyme production was significantly affected by moisture, pH, and peptone ($p < 0.05$). The $R^2$ of this model was 0.990, and the adjusted $R^2$ was 0.896. Fractional factorial design is one of the statistical methods to identify the significant medium components in an enzyme bioprocess [34]. Liu et al. [15] previously used fractional factorial design to identify the significant components of the medium. The results of the present study revealed that $2^5$ full factorial design is a good model for screening process parameters for enzyme production.

### 3.5. Optimization of enzyme production by RSM

The variables and levels were summarized in Table 4. The optimum concentration of significant factors such as moisture, pH, and peptone on fibrinolytic enzyme production was evaluated by CCD and RSM (Table 5). The model *F*-value of 157.30 implied that the model was highly significant ($p < 0.0001$). In this second-order model, the model terms such as A, C, AB, AC, BC, $A^2$, and $B^2$ were significant. The *F*-value of lack of fit was 2.04 and was nonsignificant (Table 6). The predicted $R^2$ of this model was 0.9609, and this value was in reasonable agreement with the "adjusted $R^2$" of 0.9867. The signal-to-noise ratio of this model is 41.847, which indicated an adequate signal. The $R^2$ value of this model explains 96% variability in the model. Final equation in terms of coded factors:

$$\begin{aligned}\text{Fibrinolytic activity}(Y) &= +5555.15+95.01A+40.75B+193.02C+1089.88AB-854.37AC \\ &+ 618.63BC+172.56A2-758.17B2+61.19C2\end{aligned} \quad \text{[Equation 4]}$$

where A is moisture, B is pH, and C is peptone.

**Table 4**
Experimental design and results of CCD for the production of fibrinolytic enzyme by *B. halodurans* IND18.

| Variables | Symbol | Coded values | | | | |
|---|---|---|---|---|---|---|
| | | -α | -1 | 0 | +1 | +α |
| Moisture (%) | A | 73.18 | 80 | 90 | 100 | 106.82 |
| pH | B | 7.32 | 8 | 9 | 10 | 10.68 |
| Peptone (%) | C | 0.33 | 0.5 | 0.75 | 1 | 1.17 |

**Table 5**
Response of CCD for fibrinolytic enzyme production.

| Run | Moisture | PH | Peptone | Enzyme activity |
|---|---|---|---|---|
| | (A) | (B) | (C) | (U/g) |
| 1 | 1(100) | 1(10.0) | -1(0.5) | 6063 |
| 2 | 0(90) | 0(9.0) | 0(0.75) | 5437 |
| 3 | -1(80) | -1(8.0) | 1(1.0) | 6609 |
| 4 | 0(90) | 0(9.0) | 0(0.75) | 5676 |
| 5 | -1(80) | -1(8.0) | -1(0.5) | 5720 |
| 6 | 1(100) | -1(8.0) | 1(1.0) | 5625 |
| 7 | -1(80) | 1(10.0) | 1(1.0) | 5617 |
| 8 | 1(100) | 1(10.0) | 1(1.0) | 6009 |
| 9 | 0(90) | 0(9.0) | 0(0.75) | 5676 |
| 10 | 1(100) | -1(8.0) | -1(0.5) | 5170 |
| 11 | 1.682(106.82) | 0(9.0) | 0(0.75) | 5790 |
| 12 | -1(80) | 1(10.0) | -1(0.5) | 2720 |
| 13 | -1.682(73.18) | 0(9.0) | 0(0.75) | 6354 |
| 14 | 0(90) | 0(9.0) | 0(0.75) | 5512 |
| 15 | 0(90) | 0(9.0) | 0(0.75) | 5602 |
| 16 | 0(90) | 1.682(10.68) | 0(0.75) | 3654 |
| 17 | 0(90) | 0(9.0) | 1.682(1.17) | 6054 |
| 18 | 0(90) | 0(9.0) | 0(0.75) | 5418 |
| 19 | 0(90) | -1.682(7.32) | 0(0.75) | 3225 |
| 20 | 0(90) | 0(9.0) | -1.682(0.33) | 5460 |

Three-dimensional (3D) response surface plots allowed direct visualization of interaction between each factors. These graphs were generated for the combination of two factors, while keeping the third factor at its middle point levels. The 3D plot inferred that the interactions among moisture, pH, and peptone were shown in Fig. 3a, b, and c. The response surface plot indicated that the fibrinolytic enzyme increased with increase of pH and peptone. A perturbation plot analysis showed that the increased concentration of peptone enhanced fibrinolytic enzyme production (figure not shown). Enzyme production decreased at higher moisture level of the medium. The optimal levels of the significant variables were 80% moisture, pH 8.32, and 1.0% peptone for the maximum fibrinolytic enzyme production. The predicted fibrinolytic activity was estimated to be 6876 U/g. To validate the predicted result, experiments were performed using the optimized conditions and obtained 6851 U/g enzyme activity. The experimental value (6851 U/g) was in good agreement with that of the predicted response (6876 U/g) and validated the model design. The RSM mediated optimized medium increased 2.5-fold on fibrinolytic enzyme production than no optimized medium.

Optimization of parameters for the development of economically feasible bioprocess adds advantages. RSM is one of such experimental designs by the combination of statistics and mathematics that have played a major role in the field of bioprocess engineering. Designing and analysis of experiments by statistical methods analysing the significance

**Table 6**
ANOVA for the experimental results of the CCD for *B. halodurans* IND18.

| Source | Sum of squares | df | Mean square | *F* value | *p*-Value |
|---|---|---|---|---|---|
| Model | 2.843E+007 | 9 | 3.159E+006 | 157.3 | <0.0001 |
| A—Moisture | 1.233E+005 | 1 | 1.223E+005 | 6.14 | 0.0327 |
| B—pH | 22,675.8 | 1 | 22,675.8 | 1.13 | 0.3129 |
| C—Peptone | 5.088E+005 | 1 | 5.088E+005 | 25.34 | 0.0005 |
| AB | 9.503E+006 | 1 | 9.503E+006 | 473.23 | <0.0001 |
| AC | 5.840E+006 | 1 | 5.840E+006 | 290.82 | <0.0001 |
| BC | 3.062E+006 | 1 | 3.062E+006 | 152.47 | <0.0001 |
| $A^2$ | 4.291E+005 | 1 | 4.291E+005 | 21.37 | 0.0009 |
| $B^2$ | 8.284E+006 | 1 | 8.284E+006 | 412.54 | <0.0001 |
| $C^2$ | 53,959.67 | 1 | 53,959.67 | 2.69 | 0.1322 |
| Residual | 2.008E+005 | 10 | 20,080.18 | | |
| Lack of fit | 1.348E+005 | 5 | 26,956.46 | 2.04 | 0.2261 |
| Pure error | 66,019.5 | 5 | 13,203.9 | | |
| Cor total | 2.863E+007 | 19 | | | |

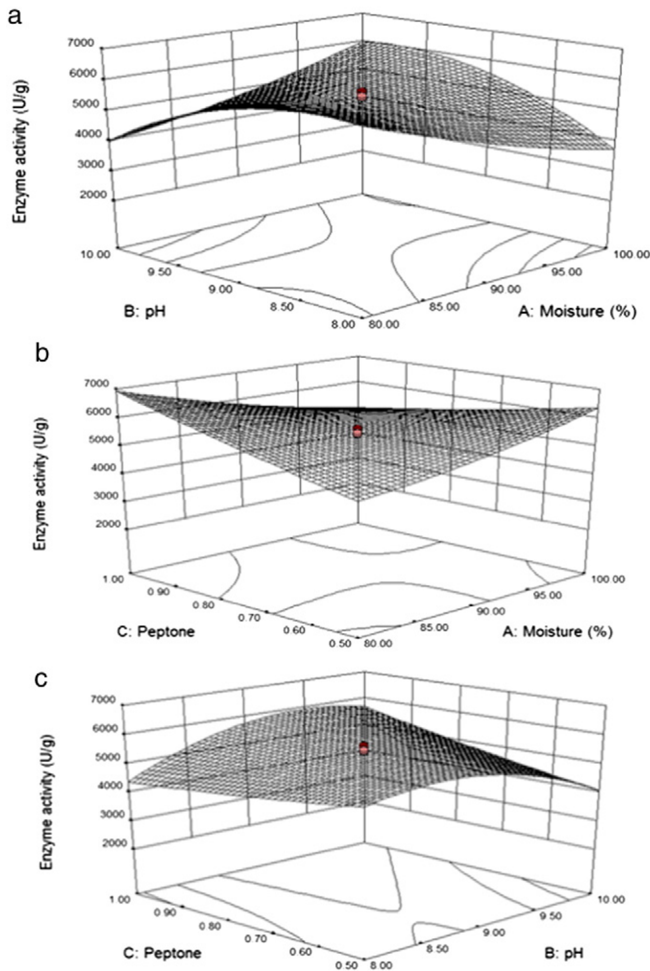


**Fig. 3.** 3-D surface plots showing the effect of interactions of various components considered for the optimization by RSM (a) moisture vs pH; (b) moisture vs peptone; (c) pH vs peptone.

of the model experiments are the major steps in enzyme bioprocess [35]. RSM allows rapid screening of various factors simultaneously with minimum cost. Earlier, RSM-mediated optimization of fibrinolytic enzyme production was applied for the evaluation of the interactive effect of casein, pH, and ammonium sulphate for *Bacillus* sp. strain AS-S20-1 [16]. Another study reports optimization of medium components such as soy peptone, calcium chloride, and yeast extract from *Bacillus natto* NLSSE using CCD and RSM to enhance fibrinolytic enzyme production [15]. The results of the present study revealed that two-level full factorial design was ideal for the screening of variables, and RSM was found to be efficient for the determination of optimized concentration of factors.

**Table 7**
Summary of the purification of the fibrinolytic enzyme from *B. halodurans* IND18.

| Procedure | Total activity (Units) | Total protein (mg) | Specific activity (U/mg) | Purification (Fold) | Yield (%) |
|---|---|---|---|---|---|
| Crude enzyme | 90,225 | 645 | 139.8 | 1 | 100 |
| Ammonium sulphate fraction | 49,030 | 275 | 178 | 1.27 | 54.3 |
| DEAE-cellulose | 18,418 | 36.7 | 501.8 | 3.6 | 20.4 |
| Casein-agarose | 11,736 | 9.2 | 1275 | 9.124 | 13 |

### *3.6. Purification of fibrinolytic enzyme*

In the present study, the fibrinolytic enzyme was initially precipitated with ammonium sulphate (70%) and further purified by DEAE-cellulose and casein-agarose affinity column chromatography. The ammonium sulphate fraction (70% saturation) showed 49,030 units of enzyme activity with 54.3% yield, and purification achieved was 1.27-fold. The specific activity of DEAE-cellulose column chromatography purified fractions was observed to be 178 U/mg protein. The casein-agarose affinity chromatography fractions showed a major peak associated with fibrinolytic enzyme activity. The specific activity of this step achieved was 1275 U/mg protein with 13% yield. The purification procedure was elaborated in Table 7. In the present study, the homogeneity of the purified fibrinolytic enzyme was tested by SDS-PAGE. The purified enzyme migrated as a single band with an apparent molecular weight of 29 kDa (Fig. 4a and b). Likewise the fibrinolytic enzyme isolated from *Bacillus* sp. strain DJ-4. showed an apparent molecular mass of 29 kDa [27].

### *3.7. Properties of fibrinolytic enzyme*

In the present study, the optimal pH for hydrolysis of fibrin was 9.0, and enzyme activity decreased rapidly at levels below pH 6.0. As shown in Fig. 5a, the enzyme was very stable at pH 9.0 at 37°C for 1 h and enzyme stability slightly decreased above pH 9.0. In accordance to this, the fibrinolytic enzyme isolated from *B. amyloliquefaciens* DC-4 was highly active at pH 9.0 [36]. The optimum pH of the isolated fibrinolytic enzyme in the present study was higher than that of fibrinolytic enzyme from *Bacillus* sp. KA38 [37] and *B. subtilis* [38]. The maximal fibrinolytic enzyme activity was observed at 60°C and decreased abruptly above this temperature and was stable up to 50°C for 1 h (Fig. 5b). This result was in agreement with the properties of fibrinolytic enzyme from *Bacillus* sp. KDO-13 [39], and the enzyme isolated in the present study was highly stable than fibrinolytic enzyme from *Bacillus* sp. strain DJ-4 [27]. The fibrinolytic enzyme produced by *B. halodurans* displayed high activity on the ions such as $Ca^{2+}$, $Mg^{2+}$, $Co^{2+}$, and $Na^{+}$, and the relative enzyme activity was 103%, 102%, 104%, and 101%, respectively. However, the ions such as $Cu^{2+}$, $Hg^{2+}$, $Fe^{2+}$, and $Zn^{2+}$ inhibited enzyme activity, and the relative enzyme activity was 57%, 83%, 61%, and 89%, respectively. This result was in agreement with fibrinolytic enzyme from *Bacillus* sp. KDO-13 [39], and fibrinolytic enzyme activity was enhanced by $Ca^{2+}$ and $Cu^{2+}$ ions, respectively. The inhibitory effect of $Cu^{2+}$, $Fe^{2+}$, and

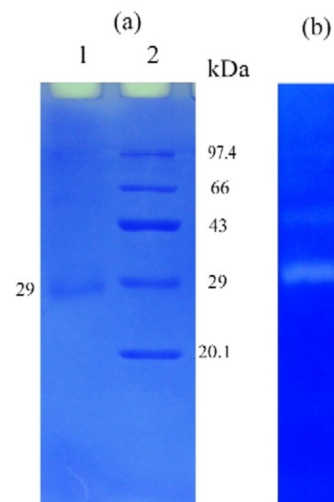


**Fig. 4.** Fibrinolytic activity of SDS-PAGE separated fibrinolytic enzyme. (a) SDS-PAGE analysis of the purified fibrinolytic enzyme; (b) fibrinolytic activity appeared as clear zone after stained with CBB.

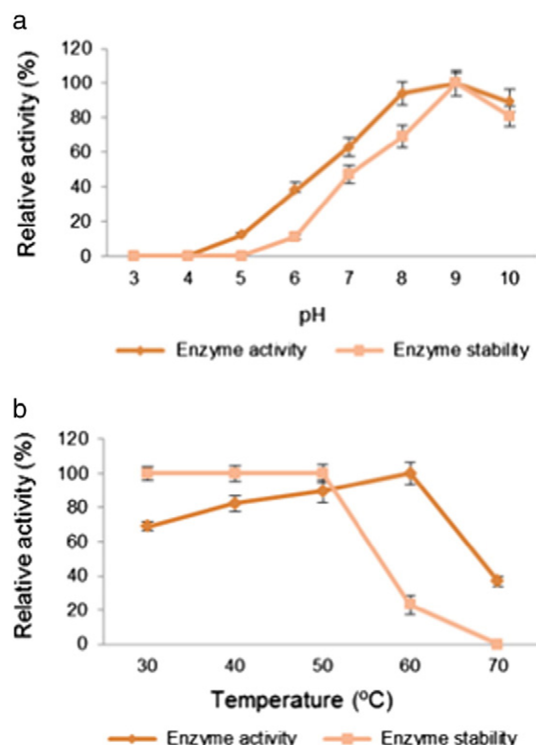


**Fig. 5.** a: Effect of pH, and b: temperature on fibrinolytic activity and stability. Relative activity is expressed as the percentage of the maximum (100% of enzyme activity).

$Zn^{2+}$ was found to be similar to that of fibrinolytic enzyme from *B. subtilis* DC 33 [40]. The fibrinolytic enzyme from *B. halodurans* dissolved human blood clot rapidly (Fig. 6). These *in vitro* studies could be useful to make this fibrinolytic enzyme as the effective thrombolytic agent. Recently, the *in vitro* blood clot lytic activity of fibrinolytic enzyme was reported from *Bacillus* sp. strain AS-S20-1 by Mukherjee and Rai [16] and from *B. subtilis* ICTF-1 by Mahajan et al. [17]. The *in vitro* clot lysis effect of the present study showed that the fibrinolytic enzyme activity is significantly higher than *Bacillus* sp. strain AS-S20-1 and *B. subtilis* ICTF-1. This enzyme directly digested the fibrin clot and also had plasminogen activator activity (Fig. 7). On plasminogen-rich plate, the fibrinolytic enzyme showed more activity than plasminogen-free plate. The fibrinolytic enzyme isolated from *B. subtilis* DC 33 had plasminogen activator activity, which was reported by Wang et al. [40]. The present study stated the ability of fibrin degradation from *B. halodurans* enzyme by activating plasminogen and also direct clot lysis.

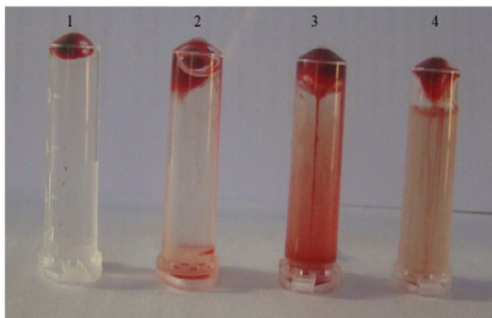


**Fig. 6.** Effect of *B. halodurans* IND18 fibrinolytic enzyme on human blood clot. 1: control; 2: 20 μg enzyme; 3: 30 μg enzyme; 4: 250 units streptokinase.

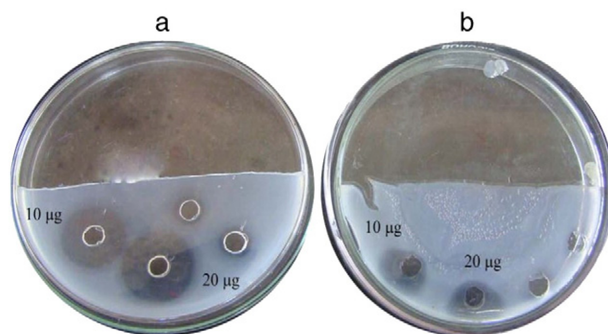


**Fig. 7.** Effect of *B. halodurans* strain IND18 fibrinolytic enzyme on plasminogen rich and plasminogen free plates. a: Fibrinolytic enzyme (10 μg and 20 μg) was dropped on both plasminogen-rich, and b: plasminogen-free plates. Enzyme activity appeared as a clear zone of degradation on these plates.

## 4. Conclusions

The bacterial isolate used agro-industrial residues for the production of fibrinolytic enzyme in SSF. The statistical tool such as two-level full factorial design, CCD, and RSM are efficient and time-saving process. The potent fibrinolytic enzyme was isolated from *B. halodurans* IND18, and it digested fibrin net of blood clot rapidly. These kinds of studies could be useful to utilize the agro-residues and to develop potent thrombolytic agents.

## Financial support

The authors extend their sincere appreciation to the Deanship of Scientific Research at King Saud University for its funding this Prolific Research Group (PRG-1437-28).

## Conflict of interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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