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A STATISTICAL AND DOWNSTREAM APPROACH FOR THE IMPROVEMENT OF PROTEASE PRODUCTION FROM *BACILLUS TOYONENSIS* VKB5 ISOLATED FROM *ACTINIDIA DELICIOSA*

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ABSTRACT

Endophytes are endosymbiotic microbes that reside inside the plant without affecting the host. *Bacillus* spp., are known to produce highly active extracellular proteases. This study aims for the optimization and partial purification of fibrinolytic enzyme production from *Bacillus toyonensis* VKB5 isolate from the fruit *Actinidia deliciosa.* Optimization studies on medium components show that maximum enzyme production can be achieved in alkaline conditions of pH 8 with nutrient sources mannitol, yeast extract and ammonium chloride with 1% (w/v) concentration. The statistical optimization studies using Plackett-Burman and Response surface methodology determines that the interaction of yeast extract with the mannitol and ammonium chloride enhances enzyme production up to 2 fold. The purification of the enzyme from ammonium sulfate to gel filtration step describes that purity of protein enhance up to 7.4 with 68.9% recovery as compared to the crude enzyme. The molecular weight was determined to be 21.9 kDa using Sodium Dodecyl Sulphate-Polyacrylamide gel electrophoresis (SDS-PAGE). The purity of the protease was analyzed using High-performance liquid chromatography (HPLC). Further studies on the effect of temperature, pH, inhibitors, detergents and metal ions confirm that the purified enzyme could be alkaline serine protease that can withstand thermal conditions up to 60 °C. The study concludes that the purified enzyme showing fibrinolytic activity having strong stability which can be further exploited for medicinal purpose.

Keywords: Endophytes; Plackett-Burman; Response Surface Methodology; SDS-PAGE; HPLC

INTRODUCTION

Fibrinolytic enzymes isolated from *Bacillus* sp., in particular, are considered to have high efficiency in plasmin activation, high substrate specificity to fibrin and is safe for humans (**Xin *et al.*, 2018**). *Bacillus* produces a wide variety of fibrinolytic enzymes such as subtilisin DJ-4 and subtilisin DFE from *Bacillus subtilus,* (**Kim & Choi, 2000**) and Nattokinase from *Bacillus natto* (**Sumi *et al.*, 1990**).
A leading contributor to the burden of non-communicable diseases is cardiovascular system diseases. The Global Burden of Diseases, Injuries, and Risk Factors Study implicated coronary heart disease and stroke as the cause of 7.0 million and 5.9 million deaths, respectively **(Raskob, 1911**). Thrombosis is the common underlying pathology of cardiovascular disorders. The failure of dissolution of a blood clot due to an imbalance in the homeostasis of the coagulation system leads to thromboembolic diseases. (**Kovalenko *et al*., 2017**) Fibrinolytic therapy is thus an effective remedy for the disorders related to a lack of fibrin clot degradation. Direct plasminogen activators such as tPA (Alteplase or Activase), Reteplase or Retavase(r-PA), streptokinase, u-PA, pro-urokinase (pro-uPA) are currently in clinical use as fibrinolytic agents **(Adivitiya & Khasa, 2017; Kotb, 2014**).
Newer plasmin variants and targeted delivery techniques are future approaches in fibrinolytic therapy. In addition to these, fibrinolytic agents of microbial or plant origin are the current highlights in terms of novelty (**Adivitiya & Khasa, 2017; Kotb, 2013)**. Microbial fibrinolytic enzymes have been a focal point of interest in recent years due to their rapid growth, diversity, ease of genetic manipulation, convenience and economy of their production. Most of the microbial strains capable of fibrinolytic enzyme production belong to the *Bacillus* genus. Strains belonging to *Pseudomonas*, *Streptomyces*, *Serratia*, *Actinomyces*, *Staphylococcus*, *Penicillium*, *Aspergillus* genera too have been reported to produce this enzyme.(**Mushtaq & Jamil, 2012; Taneja *et al*., 2017**).
Endophytic microorganism resides in the host plant and does not exhibit any sign of their presence. Endophytes portray a symbiotic relationship with the host plant, as they increase the capabilities of stress tolerance, defend against pathogens and thus increase the overall fitness of the host plant by producing many novel bioactive compounds that can be put to human use (**Meshram & Saxena, 2016; Nair & Padmavathy, 2014**). Predominantly, endophytic fungi have been studied for their fibrinolytic potential. Endophytic fungi such as *Cladosporium* sp., *Clonostachys* sp., *Fusarium* strain CPCC 480097, *Verticillium* sp., *Lasiodiplodia* sp. and *Fusarium* sp. BLB has been demonstrated to have fibrinolytic activity. Studies on the fibrinolytic capabilities of endophytic bacteria seem to be limited to a few organisms such as *Paenibacillus polymyxa* EJS-3 (**Lu *et al.*, 2010**). Kiwi (*Actinidia deliciosa)* was chosen as the fruit to isolate endophytic bacteria from, based on studies that indicate the fibrinolytic activity of enzymes derived from this fruit (**Habib & Saad, 2013; Kim *et al.*, 1996; Velusamy *et al.*, 2016**).
The maximum production of the enzyme from any source necessitates optimization of the growth media to reduce cost. The carbon, inorganic and organic nitrogen sources, as well as the growth conditions such as the pH, temperature, incubation time, affect the yield, the production pattern as well as the quality of the enzymes produced, hence need to be optimized. Statistical models such as Plackett-Burman (PB) and Response surface methodology (RSM) are reliable methods to gauge the interaction between different parameters of growth suitable to the organism and its effect on enzyme yield (**Singh & Bajaj, 2015**).
The present study aimed to search for a fibrinolytic enzyme produced by an endophytic bacterium isolated from fruit samples. An optimized media suitable to the organism isolated, *Bacillus* sp., was developed and purification to enhance the process applications was carried out. Characterization of the enzyme to study the effects of pH, metal ions, inhibitors, the temperature on its enzyme activity and stability were carried out.

MATERIALS AND METHODS

Sample collection

Actinidia deliciosa (Kiwi) was obtained from a fruit market in Vellore, Tamil Nadu. Around five fruits were bought in normal conditions. Fruits were collected in a sterile bag and transported to the laboratory under normal environmental conditions.

Surface sterilization

The fruit *A. deliciosa* was washed in running tap water to remove superficially present unwanted dust particles. Further, this procedure was followed by washing the fruit with 70% ethanol for five minutes to remove microorganisms. Subsequently, the fruit was rinsed in 0.4% sodium hypochlorite containing 1% Tween20 which helps in removing proteinaceous substances. Then the fruit was washed thrice, in sterile distilled water, for five minutes and air-dried. All sterilization procedures were carried out aseptically (**Heun Hong & Gross, 1998; Meshram & Saxena, 2016**).

Isolation of bacteria

Thin inner peels of the surface-sterilized fruit were sliced using a sterile blade and impregnated on nutrient agar plates to encourage the growth of the endophytic bacteria present on the inner skins. These plates were then incubated at 37 °C for 24 h. Distinct colonies found growing on the plate were further cultured separately for enzyme production screening using the streaking technique.

Screening of bacteria by qualitative analysis of protease activity

The Pure cultured bacterial colonies were screened for protease activity using skim milk agar plate assay and fibrin degradation using fibrin plate assay. For the protease activity, the colonies were streaked on skim milk agar plates (composed of (w/v) peptone (5%), yeast extract (1.5%), Skim Milk (1.5%) and Agar(15%)) containing 5% NaCl and incubated for 24 h at 37 °C. The appearance of a clear zone indicates the degradation of casein which is considered as positive for protease activity (**Amid *et al*., 2019**).
To understand the degradation of a fibrin clot, the well-established fibrin plate method was followed. The plate composed of 0.5% fibrin in 10 mL agarose (1%) solution. To which 0.1 mL of 100 NIHU/mL of thrombin was added and allowed to solidify. Wells were punched using a suction syringe with a 2 mm diameter. Around ten µl supernatant of each isolate was added to the wells. Further, the plates were allowed to incubate for 18 h at 37 °C. The appearance of a clear zone around the wells indicated the degradation of fibrin. Isolates that produced higher degradation activity were selected for further studies. (**Vijayaraghavan & Vincent, 2014; Wang *et al*., 2012; Zhang *et al*., 2015**).

Molecular and morphological analysis

To understand the morphological characteristics of the screened isolate, Gram staining was performed. The Genomic DNA of the selected strain was extracted from cells of a 24 h culture. The 16S rDNA sequencing of the selected strain was PCR amplified using universal primers in the ABI VERTIC thermocycler. The PCR product was purified with a base pair of approximately 1477 bp and was sequenced by using the upstream primer 27F and downstream primer 1492R. PCR analysis was performed using the ABI PRISM® BigDye 2TM terminator cycle kit. The resultant product was electrophoresed in an ABI3730XL automated DNA sequencing system. The obtained nucleotides were then subjected to a homology search using the BLAST program of the National Centre for Biotechnology Information (NCBI) (**Agrebi *et al*., 2009; Mahajan *et al*., 2012**).

Quantitative analysis

A standard protease assay was performed to quantify the enzyme activity of the isolates. The crude enzyme was obtained by centrifuging the bacterial isolates' broth at 10,000 rpm for 10 minutes and taking the supernatant. A mixture of 2.5 mL of 1% fibrin (used as substrate) in 0.1M Tris HCl buffer (pH 7.8) and 0.5 mL of crude enzyme solution was incubated at 37 °C for 30 minutes. The reaction was stopped by the addition of 4.5 mL of Trichloroacetic acid (TCA) (110 mM TCA, 220 mM Sodium Acetate and 0.33M of Acetic Acid) and the mixture was centrifuged for 10 min at 10,000 rpm. The absorbance of the supernatant obtained was measured at 280nm in a UV-spectrophotometer and was used to determine the release of free amino acids, namely tyrosine. A standard graph of tyrosine was used to estimate the protease activity. A single unit of protease activity (U) was defined as the amount of the enzyme that releases one µM of tyrosine equivalent per ml of the enzyme per minute (**Chandrasekaran *et al*., 2015; Majumdar *et al*., 2015**).

Classical method of media optimization (OFAT-One Factor at A Time)

The classical method was used to optimize the growth medium for bacteria VKB5 to screen for factors that would affect the production of fibrinolytic enzymes. In this method, only one factor/variable was varied in each run while keeping the other factors/variables constant. It is used in the initial stages of the formulation of a completely unknown media. The simplicity and ease of the classical method make it a valuable screening tool (**Singh *et al*., 2017**). A preliminary study was conducted to establish the favorable nutrient sources of carbon (Dextrose, Starch, Sucrose, Maltose, Fructose, Mannitol), Organic nitrogen (Yeast Extract, Peptone, Skim Milk Powder, Soybean Meal) and Inorganic nitrogen (Ammonium Chloride, Sodium Carbonate, Monosodium phosphate, Potassium Nitrate) (**Mukherjee & Rai, 2011**). 1% (w/v) of each of the nutrient sources (carbon, nitrogen and inorganic nitrogen) was individually added to separate test tubes and sterilized, followed by inoculation with 100 µL in the culture broth. The test tubes were incubated for 48 h, followed by a protease assay to measure the enzyme activity of each factor, which would signify the optimal growth of the bacteria in the corresponding nutrient source. Physical factors such as Incubation time or Fermentation period (48 to 96 h), pH(4, 6, 7, 8, 9, 12), temperature (37, 40 and 60 °C) and Rate of agitation (rpm) (50, 100, 150, 200) were also evaluated to select the factors that promote greater enzyme yield (**Vijayaraghavan *et al*., 2015**). The optimal factors showing high enzyme activity was chosen for further statistical optimization studies.

Statistical Analysis

Media optimization by PB Method

The individual effect of each variable from the nutrient and physical factors selected from OFAT was evaluated using the PB method. It relays the variable that is most significant to the medium and protease production. Protease assay was carried out for each run. Seven factors were chosen for optimization, Mannitol (carbon source), Yeast Extract (organic nitrogen source), Ammonium chloride (inorganic nitrogen source), pH of the medium, Temperature of incubation (°C), incubation time (h), agitation rate (rpm). Placket Burman is considered as a preliminary method of optimization as it tests only two levels, the high (+1) and low (-1), of each factor as given in Table 1.

Table 1 Factors selected for PB Design

Factors	Units	Coded levels	
		Low	High
Mannitol (A)	% (w/v)	0.5	1
Yeast Extract (B)	% (w/v)	0.1	1
NH_4Cl (C)	% (w/v)	0.05	0.25
pH (D)		4	7
Temperature (E)	°C	25	37
Fermentation period (H)	H	72	96
Agitation (I)	Rpm	100	150

The linear and curvature effects of the variables were evaluated by running a center point. The experimental design is based on the first-order polynomial model:

$$Y = \beta_0 + \Sigma\, \beta_i X_i \quad (1)$$

Here, Y signifies the response (the total enzyme activity in U/mL) (Table 2), β_0 is the intercept and β_i is the linear coefficient, and X_i is the level of the independent variable.

The drawback of this model is that it does not describe the interaction amongst factors and it is only used to evaluate and screen the most important factors that influence the response (Table 2). Regression analysis of the variables helped in the selection of the most significant factors for fibrinolytic enzyme production; these were further optimized by the Response Surface Methodology(RSM) (**Mukherjee & Rai, 2011**).

Optimization by response surface methodology

The variables selected after PB optimization were then evaluated using RSM. RSM helps in understanding the optimum concentration of each of the variables of the medium and their combined effect in the response (enzyme yield) produced (**Vijayaraghavan & Vincent, 2014**). Protease assay is performed for each run to determine the series with the optimal concentrations of the variables to support high yields of the fibrinolytic enzyme. Statistical software 'Design-Expert 10' was used for the design and analysis of the experimental data.
Central Composite Design (CCD) was used to optimize the conditions of fermentation further. CCD consisted of three crucial independent variables: Carbon Source (Mannitol) (C1), Organic Nitrogen Source (Yeast Extract) and (iii) Inorganic Nitrogen Source (Ammonium Chloride). These factors each had five levels (−α, −1, 0, +1, +α) (Table 43) and consisted of 20 experimental runs (6 central,8 factorial, and 6 axial points) as given in Table 3.

Table 2 PB Design Table with corresponding enzyme activity

Run	Mannitol %	Yeast Extract %	NH_4Cl %	pH	Temperature °C	Incubation h	RPM	Enzyme Activity(U/mL)
1	1	1	1	4	25	72	150	418.29
2	1	1	0.1	4	25	96	100	520.5
3	1	1	0.1	7	37	96	100	432.98
4	0.1	1	1	7	25	72	100	448.19
5	0.1	0.1	0.1	4	25	72	100	94.24
6	0.1	0.1	0.1	7	25	96	150	107.3
7	1	0.1	1	7	37	72	100	422.16
8	0.1	1	0.1	7	37	72	150	106.13
9	0.1	0.1	1	4	37	96	100	110.75
10	1	0.1	0.1	4	37	72	150	133.3
11	1	0.1	1	7	25	96	150	147.16
12	0.1	1	1	4	37	96	150	545.6

Table 2 Individual Variables and their Coded Values for Central Composite Design

Factors	Units	Coded levels				
		−α	**−1**	**0**	**+1**	**+α**
Mannitol	% (w/v)	-0.206807	0.1	0.55	1	1.30681
Yeast Extract	% (w/v)	-0.206807	0.1	0.55	1	1.30681
NH_4Cl	% (w/v)	-0.206807	0.1	0.55	1	1.30681

The data obtained was fit as a second-order polynomial regression equation that includes both the individual effect and the cross effect of each variable.

$$Y = a_0 + \sum_{i=1}^{3} a_i C_i + \sum_{i=1}^{3} a_{ii} C_i^2 + \sum_{i=1}^{2} \sum_{j=i+1}^{3} a_{ij} C_{ij} \quad (2)$$

where Y is the response (total enzyme activity (U/mL)), a_0 is the intercept term, a_i is the linear effect, a_{ii} is the square effect, a_{ij} is the interaction effect, and Ci and Cj the variables. The equation was used to optimize the values of each independent parameters for the response **(Mukherjee & Rai, 2011; Vijayaraghavan & Vincent, 2014**).

Table 3 Central Composite Design: Experimental Design and Response (Y) in Enzyme activity (U/mL)

Run	Mannitol %	Yeast Extract %	NH4Cl %	Enzyme Activity U/mL
1	0.55	0.55	0.55	897.7
2	1	1	1	469.1
3	1	1	0.1	697.2
4	1	0.1	1	135
5	0.55	1.30681	0.55	969.1
6	0.1	1	0.1	928
7	0.55	-0.206807	0.55	100.4
8	0.55	0.55	0.55	895.6
9	-0.206807	0.55	0.55	535.6
10	0.1	1	1	891.8
11	0.1	0.1	1	243.1
12	0.55	0.55	1.30681	620.4
13	1.30681	0.55	0.55	107.7
14	0.55	0.55	0.55	897.2
15	0.55	0.55	0.55	891.2
16	0.55	0.55	-0.206807	769.1
17	0.55	0.55	0.55	897.2
18	1	0.1	0.1	221.8
19	0.1	0.1	0.1	106.4
20	0.55	0.55	0.55	897.1

Purification and characterization of the Fibrinolytic Enzyme

To the cell-free supernatant obtained, 60% (w/v) ammonium sulfate was added and incubated overnight on a magnetic stirrer at 4 °C. The salt precipitated proteins were obtained in the form of pellets upon centrifugation at 10,000 rpm for 10 minutes. The pellet was dissolved in a small amount of 20 mM Tris-HCl buffer (pH 7.4) (**Majumdar *et al*., 2015**). The dissolved pellet solution is dialyzed in a dialysis membrane 60 (>30,000 da) against 20 mM Tris-HCl buffer solution of pH 7.4 for 12 hours at 4 °C, with a fresh buffer change every two hours.

The concentrated protein solution obtained after dialysis was loaded to a DEAE Cellulose 52 Anion-Exchange Column after equilibration with the Tris-HCl Buffer. Elution was conducted in a stepwise manner with a NaCl gradient (0.1 to 1 M) as the elution buffer at regular intervals with a flow rate of 2 mL/min. The chromatography method was performed at 4 °C to prevent the denaturation of the enzyme. The protease activity of each fraction was measured and the fractions with the highest activity were pooled together to be purified further through the gel filtration chromatography process (**Balaraman & Prabakaran, 2007; Paik *et al*., 2004**).

Sephadex G-100 column was used for the Gel Filtration process of the pooled fractions at 4 °C. The column was equilibrated with 20mM Tris-HCl buffer (pH 7.4). The sample was loaded into the column and eluted using the same concentration of Tris-HCl Buffer. Fractions (2ml each) were collected at regular intervals. The protein concentration of the fractions was determined using the Bradford method with Bovine Serum Albumin (BSA) as the standard **(Walker & Kruger, 2003**). The absorbance was taken at 595 nm using a UV-Spectrophotometer. Protease activity further determined the active fractions upon completion of the process and these fractions were pooled together resulting in the final enzyme sample. The final sample was lyophilized to concentrate on a lyophilizer at −80 ° C (**Paik *et al*., 2004; Wu *et al*., 2009**).

SDS PAGE

The molecular weight and the purity of the lyophilized active samples were assessed by running a Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS PAGE). The enzyme solution was prepared by dissolving the enzyme in the Tris-HCl buffer (pH 6.8) with 0.5% of SDS and mercaptoethanol. The enzyme solution and standard protein markers for molecular weight were run alongside each other on a PAGE gel which was subsequently silver-stained for visualization (**Balaraman & Prabakaran, 2007; Wu *et al*., 2009**).

HPLC (High-Performance Liquid Chromatography)

HPLC was used to determine the purity of the protein. A GF-250 column was used to carry out the process. The flow rate was maintained at 2 mL/min, Mobile phase used was Acetonitrile: Water (80:20). A UV 254 detector was used. The retention time of any molecule is said to be inversely proportional to the logarithm of the molecule's size which is related to the molecular weight. The experiment was performed in WATERS 2487 in a binary HPLC Pump 1525.

Biochemical Characterization

The effect of pH on the extracellular protease was studied with varying pH ranging from 3 to 12 using various buffers (20 mM): sodium acetate buffer (pH 3-6); Tris HCl buffer (6.5-8.8); Phosphate buffer (9-12). The enzyme activity was estimated by performing the protease assay after incubating the purified enzyme in the various buffers for four hours at room temperature. Similarly, the effect of temperature was also studied by incubating the purified protein at a different temperature ranging (4, 25, 37, 45 and 60 °C) for four hours for stability analysis and the enzyme assay was subsequently performed.

Further, the effect of various metal ions and inhibitors on enzyme activity was also studied. Metal salts such as $FeSO_4$, $MgSO_4$, $MnSO_4$, $ZnSO_4$, $CaCl_2$, $CuSO_4$; inhibitors such as DTPA (Diethylenetriamine pentaacetic acid), EDTA (Ethylenediaminetetraacetic acid), DTT (Dithiothreitol), SDS (Sodium dodecyl sulfate), IPTG (Isopropyl β-D-1-thiogalactopyranoside), PMSF (Phenylmethylsulfonyl fluoride) were incubated with the purified protein for 4 hours at 37 °C. Incubation was followed by protease assay to determine the components that may enhance or inhibit the activity of the fibrinolytic enzyme.

RESULTS

Isolation, screening and identification of the fibrinolytic enzyme-producing bacteria

Eight colonies were obtained after the initial incubation of the inner *A. deliciosa* peels (figure 1a). These colonies were labeled (VKB1-VKB8) and screened on skim milk agar and fibrin plates (figure 1b and c). Out of the 20 isolates (VKB1-VKB20) obtained from the colonies three of them showed proteolytic activity, as well as displayed a broad zone of degradation in the fibrin plate method, thus signifying a good fibrinolytic agent. Among the three, the quantitative enzyme activity for the isolates showed that isolate VKB5 had a significantly higher fibrinolytic activity and was hence selected for further studies. The phenotypic characterization of isolate VKB5 showed that it was Gram-positive in nature.16S rDNA gene sequencing classified the isolate as a *Bacillus toyonensis* by performing a BLAST search. The phylogenetic tree was constructed in MegaX software showing the closeness of relationship (figure 1d).

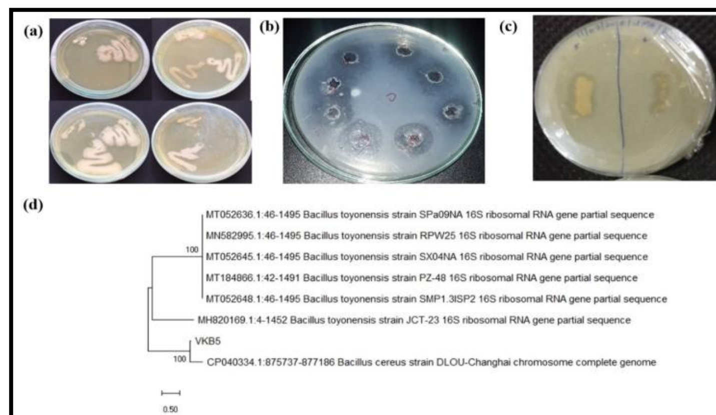

Figure 1 Purification and Screening of bacterial isolates hosting in *Actinidia deliciosa* for their protease activity. Pure culture of the isolates (a); Qualitative Screening of each isolate were performed and their zone of degradation (b and c). Phylogenetic tree constructed to understand the closeness of relationship between the isolate and other strains (d).

Preliminary fermentation studies

Preliminary optimization studies were conducted using the one-factor-at-a-time approach (OFAT). The optimum physiological and compositional conditions were determined by subjecting strain VKB5 to varying pH, agitation rates, incubation period, temperature and nutrient sources like carbon, nitrogen and metal ion supplements. Enzymes are secreted by the bacteria during the stationary phase or the late exponential phase of the bacterial growth cycle. Thus, it is necessary to monitor the culture for the maximum production of the enzyme according to the time profile. In our study, the time profile analysis showed that the production of the enzyme started at 24 h and reached a maximum of 96 h. Temperature alters the physical state of the cell membrane and can thus affect the uptake of the substrate as well as its utilization. It thus influences the extracellular secretion of the enzyme. Temperature analyses depicted significant enzyme production in the range of 4 °C - 60 °C with maximum production at 37 °C (39.183 U/mL) and very less activity at 60 °C (10.048 U/mL). The study on the effect of varying pH values indicated that maximum enzyme was produced at slightly alkaline or neutral pH at 8 while the enzyme production rapidly decreased on either side of this range. The enzyme production reduced more in acidic conditions as compared to higher alkaline conditions. The agitation rate (rpm) indicates the level of dissolved oxygen, the extent ofs mixing of the contents and the availability of nutrients in the broth. It also has direct effects on the production of the enzyme as well as cell growth. High agitation rates may lead to the destruction of the cell. Studying the effect of various agitation rates on the production of the enzyme indicated that the maximum production of the enzyme occurred at 100 rpm (12.248 U/mL).

Among the various carbon sources used in the production of the fibrinolytic enzyme, Mannitol resulted in the maximum production of the enzyme 92.459 U/mL; followed by Maltose and Glucose (figure 2a). Nitrogen source alters the pH and provides nitrogen precursors for the cellular components hence is an important factor in the stability as well as the production of the enzymes. The organic nitrogen source, Yeast Extract, resulted in maximum enzyme production of 504.038 U/mL (figure 2b), coupled with Ammonium chloride as the inorganic nitrogen source which gave a maximum enzyme production, amongst other inorganic sources, of 121.416 U/mL (figure 2c).

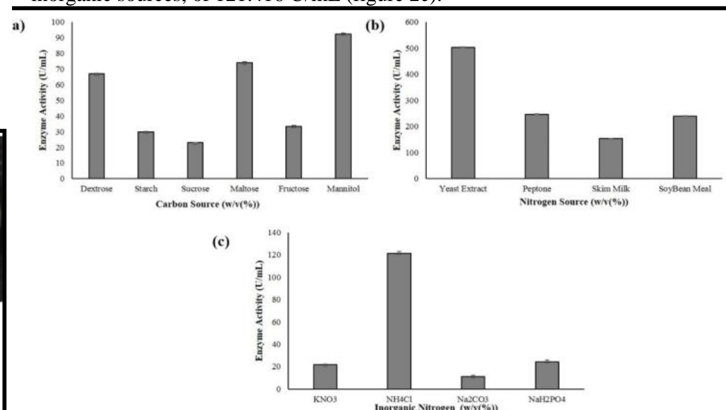

Figure 2 Optimization of Medium components for the production of fibrinolytic enzyme from the strain VKB5 using OFAT approach (w/v(%)). (a) Carbon Source; (b) Nitrogen source and (c) Inorganic Nitrogen Source.

Selection of significant variables by PB method

The significant variables required for protease production were selected based on the generated design matrix. The enzymatic responses obtained for each run consisting of different combinations of variables are shown in Table 2. The variables that had the most statistically significant effect were determined by t-test for ANOVA. Yeast extract with a probability value of 0.0102 was the most significant factor followed by NH_4Cl (0.1479) and Mannitol (0.1670) as depicted in Table 5. The lower the probability values, the more significant are the factors for the production of protease. The Model F-value of 5.35 implies that the model is significant. There is only a 2.57% chance that an F-value this large could occur due to noise. Values of probability > F being less than 0.05 indicate that the model terms are significant. In this case, factor B (Yeast Extract) is a significant model term. Hence amongst the seven variables that were screened, three (yeast extract, NH_4Cl and Mannitol) were found to have the most significant effect on protease production by VKB5. These were then selected for further optimization using RSM. The regression equation in terms of the coded factors was as follows:

$$\text{Enzyme Activity (Y)} = 290.55 + 55.18A + 121.40B + 58.14C$$

Further statistical analysis was conducted. The Predicted R-Squared value of 0.2519 is not as close to the Adj R-Squared value of 0.5428 as one might normally expect; i.e. the difference is more than 0.2. This may indicate a large block effect or a possible problem with the model and/or data. Things to be considered further are model reduction, response transformation, outliers, etc. Adeq Precision measures the signal to noise ratio. A ratio greater than 4 is desirable. The ratio in our experiment yielded a value of 6.466 indicating an adequate signal. This model can be used to navigate the design space.

Table 5 Analysis of Variance of PB design

Source	Sum of Squares	Df	Mean Square	F Value	p-value Prob > F	
Model	2.540E+005	3	84652.10	5.35	0.0257	significant
A-Mannitol	36540.20	1	36540.20	2.31	0.1670	
B-Yeast Extract	1.769E+005	1	1.769E+005	11.18	0.0102	
C-NH4Cl	40565.44	1	40565.44	2.57	0.1479	
Residual	1.265E+005	8	15811.47			
Cor Total	3.804E+005	11				

Optimization of protease production by RSM

The three significant variables that have maximum influence on the protease production, indicated by PB were further optimized using the CCD of RSM, keeping the other parameters at a fixed level. Design-Expert software was used to analyze the effective variables. Analysis of variance was performed to the experimental design used. The significant model terms are A, B, C, D, A^2, B^2, C^2, D^2, AC, BC, BD, and CD. The regression equation for the quadratic model was derived to be:

$$\text{Enzyme Activity (Y)} = 897.05 - 100.01A + 273.91B - 34.01C - 82.60AB - 51.92AC - 39.27BC - 209.92A^2 - 134.58B^2 - 78.01C^2$$

The equation depicts the variation of the enzyme activity (Y) as a function of Mannitol (A), Yeast Extract (B) and NH_4Cl (C). The model F-value of 167.80 implies the model is significant. There is only a 0.01% chance that an F-value this large could occur due to noise. Values of Probability > F-value of < 0.05 indicate that the model terms are significant. In this case, A, B, C, AB, AC, BC, A^2, B^2, C^2 are significant model terms. If values are greater than 0.1000, it would indicate that the model terms are not significant. If there are many insignificant model terms, model reduction may improve your model. The Lack of Fit F-value of 4.64 implies the Lack of Fit is significant. There is only a 0.01% chance (probability>F) that a Lack of Fit F-value this large could occur due to noise. A significant lack of fit is not appropriate since we want the model to fit. (Tab 6). Adequate precision which measures the signal to noise ratio was 34.766 for this model. A ratio greater than 4 is desirable, hence indicating adequate signal in terms of our model and verifies its users to navigate the design space. The predicted correlation coefficient (Predicted R^2) of 0.9501 is in reasonable agreement with the experimental value of 0.9875 (i.e. the difference is less than 0.2).

Table 6 Analysis of Variance for RSM design

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F	
Model	2.121E+006	9	2.357E+005	167.80	< 0.0001	significant
A-Mannitol	1.366E+005	1	1.366E+005	97.25	< 0.0001	
B-Yeast Extract	1.025E+006	1	1.025E+006	729.49	< 0.0001	
C-NH_4Cl	15797.49	1	15797.49	11.25	0.0073	
AB	54582.08	1	54582.08	38.86	< 0.0001	
AC	21569.65	1	21569.65	15.36	0.0029	
BC	12340.20	1	12340.20	8.79	0.0142	
A^2	6.351E+005	1	6.351E+005	452.15	< 0.0001	
B^2	2.610E+005	1	2.610E+005	185.83	< 0.0001	
C^2	87708.89	1	87708.89	62.44	< 0.0001	
Residual	14045.93	10	1404.59			
Lack of Fit	14015.75	5	2803.15	4.64	< 0.0001	Non-significant
Pure Error	30.18	5	6.04			
Cor Total	2.135E+006	19				

The two-dimensional and three-dimensional response surface plots for enzyme production depicting the interactive effects of the significant variables are shown in figure 4. The variation in the enzyme production when the value of each individual factor changes independent of the other factors (kept constant), is depicted in figure 3. Yeast extract was seen to have a strong positive effect on the production of the fibrinolytic enzyme, both in terms of its interactions as well as a single component.

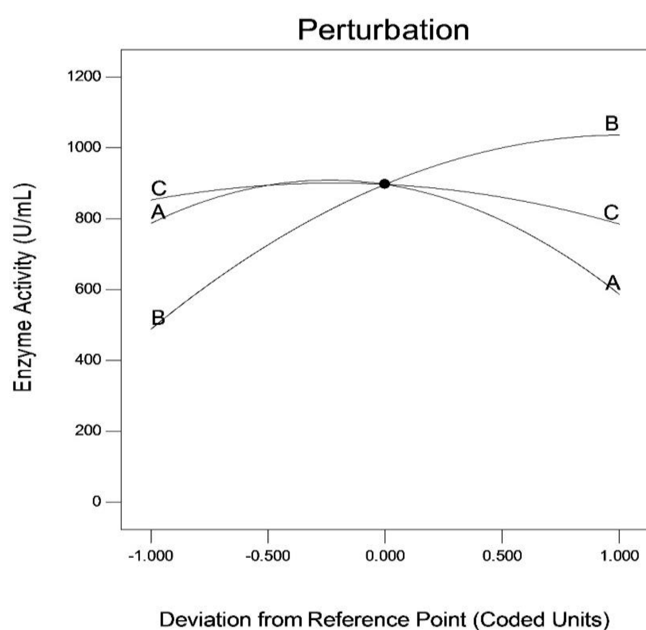

Figure 3 Perturbation curve showing the effect of independent variables on enzyme activity from their centre points. Mannitol (A); Yeast Extract (B); NH_4Cl (C).

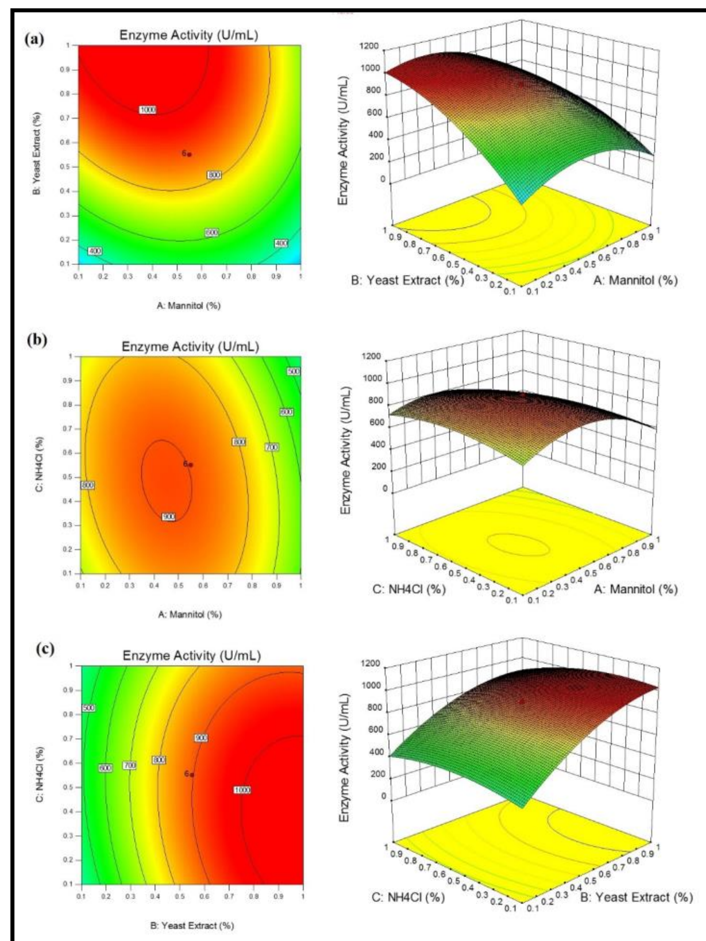

Figure 4 Response Surface of 2-D and 3-D plots of interactive effects of different independent variables on protease activity. (a) Mannitol (A) and Yeast

Extract (B); (b) Mannitol (A) and NH_4Cl (C); (c) Yeast Extract (B) and NH_4Cl (C).

Purification of the fibrinolytic enzyme

The fibrinolytic enzyme from the isolated *Bacillus toyonensis* was purified using a combination of steps ranging from ammonium sulphate precipitation to chromatographic steps. At each step, the enzyme activity was assessed to determine the product yield and recovery. As depicted in Table 7, the final enzyme recovery was found to be 68.9 % with a 7.4 -fold pure enzyme obtained. Further characterization like molecular weight determination using SDS-PAGE (figure 5a) depicted that the purified enzyme weighed 21.9 kDa. The molecular weight was calculated using graph plot Rf vs log(MW) as shown in figure 5b . Further, the single peak appearance in HPLC analysis confirms the purity of the enzyme (figure 6).

Table 7 Enzyme recovery and purification fold at each stage of Purification with their enzyme Activity.

Purification Steps	Enzyme Activity(U/mL)	Protein Estimation	Specific activity	Recovery	fold
Crude Enzyme	1290.27	462	2.79	100	1
Ammonium Sulphate Precipitation	1213.4	376	3.23	94.04	1.15
Dialysis	1122.83	99.5	11.28	87.02	4.04
Ion Exchange Chromatography	1102.36	68.3	16.13	85.43	5.78
Gel Filtration Chromatography	890.23	43.02	20.69	68.9	7.4

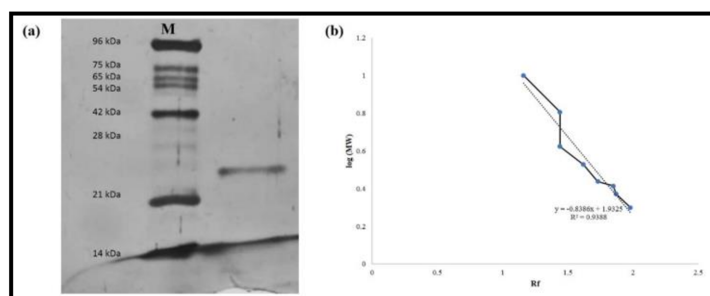

Figure 5 (a) SDS-PAGE for the protease purified (M: Marker Lane); (b) Graph plot of Rf vs log(MW).

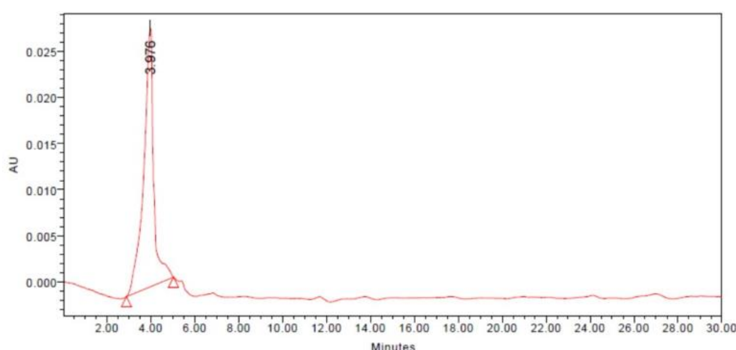

Figure 6 HPLC analysis was performed to understand the purity of an enzyme. The single peak (3.976) has appeared in 5 mins of elution determining the purity of the enzyme.

Characterization of purified fibrinolytic enzyme

Effect of pH and temperature on enzyme activity and stability

The influence of pH on the enzyme activity was determined and the results depicted that the enzyme was highly active at pH 7-8 (190 U/mL). Acidic pH conditions reduced the enzyme activity. Thus, the purified enzyme was assumed to be an alkaline protease (figure 7b). The study on the effect of temperature on the activity of the purified fibrinolytic enzyme showed that the maximum activity of the enzyme was observed at 55 °C and it decreased above this temperature (60 °C) with a relative activity of about 16.67% (163.5 U/mL). While it decreased at temperatures lower than 55 °C with a relative activity of about 22.15% (196.2 U/mL) as shown in figure 7a. as compared to the initial activity of the purified enzyme (153.405 U/mL) at room temperature.

Effect of metal ions on enzyme activity

The effect of metal ions on the activity of the purified fibrinolytic enzyme was evaluated to gain a clearer understanding of their regulatory role in enzyme activity. The purified enzyme was found to be stable and was even stimulated to have a higher catalytic effect (as compared to the initial activity of,153.405 U/mL) in the presence of a few metal ions. All the metal ions used, namely, Fe^{3+}, Ca^{2+}, Mg^{2+}, Zn^{2+} and Mn^{2+} enhanced the activity of the enzyme while the maximum activity was obtained in the presence of Cu^{2+} ions.(299.081 U/mL) as depicted in figure 7d. Copper is an important cofactor of a number of enzymes in the human body, hence the activity of our proposed fibrinolytic enzyme agent won't be subdued in the body by the various metal ions present as cofactors.

Effects of inhibitors and solvents on enzyme activity

The purified enzyme was found to be stable and even had enhanced activity in the presence of several known inhibitors. Metal chelator EDTA did not inhibit the enzyme activity, indicating that the mechanism of enzymatic activity was not dependent on cations. Other known inhibitors such as SDS, DTPA, IPTG, PMSF, and DTT showed a stimulatory effect on the activity of the enzyme. Thus, the enzyme isolated from *Bacillus toyonensis* is stable and has stimulated activity in the presence of many metal ions as well as solvents and detergents (figure 7c)

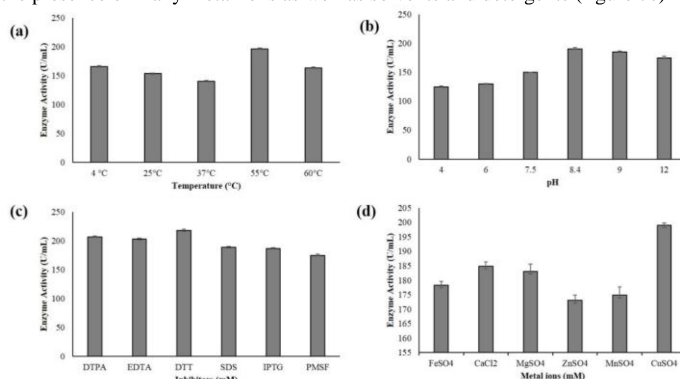

Figure 7 Effect of each environmental and interaction of chemicals to analyze the purified enzyme stability. (a) effect of temperature; (b) effect of pH; (c) effect of inhibitors and (d) effect of metal ions.

DISCUSSION

Fibrinolytic enzymes from *Bacillus sp.* have gained interest in recent times owing to the safer use and high efficiency in the fibrinolytic process. Our study resulted in the isolation, purification, and characterization of an efficient fibrinolytic enzyme from *Bacillus toyonensis* VKB5, an endophytic microbe isolated from the kiwi fruit. The organism has not been previously reported to produce fibrinolytic enzymes. Most studies center around fibrinolytic enzymes produced by *Bacillus subtilis* and *B. amyloliquefaciens* strains (**Singh & Kumar, 2017**). Endophytic organisms can range from being symptomless symbiotic in nature to be opportunistic pathogens. They have been shown to produce a wide range of useful products such as producing toxins that can discourage insect infestation. Such discoveries of interest concerning endophytes have been responsible for encouraging extensive screening of endophytes for bioactive compounds and enzymes (**Li *et al*., 2007**).

Some endophytes have also been shown to be able to synthesize the same compounds that are produced by their host plants. The endophytic fungus *Taxomyces andreanae* isolated from the *Taxus brevifolia* tree can produce the anticancer compound Taxol, for example (**Abdel-Azeem *et al*., 2019**) Similarly,our study demonstrates that the endophytic bacterial strain isolated from the kiwifruit is capable of producing fibrinolytic enzymes, similarly kiwifruit has been shown to have good fibrinolytic properties, thus the endophytic bacteria mirrors the host fruit in the production of the bioactive compounds (**Jung *et al*., 2005**). Hence, exploring the endophytic biodiversity of plants that produce bioactive compounds of human significance may result in the discovery of endophytic organisms that may aid in increasing the yield of these compounds, since microorganism sources will produce a greater output as compared to directly extracted from the plant source. Endophytic fungi have been reported to produce these fibrinolytic enzymes, while studies on bacterial species

that produce these enzymes have been limited (**Li *et al*., 2007**). The growth of the organism and thus the yield of the fibrinolytic enzyme was optimized by various steps of media optimization such as PB and RSM to achieve enhanced enzyme activity and yield.

The activity of our enzyme mirrors the enzyme isolated from *Bacillus subtilis* KCK-7 in its enhancement of activity in the presence of Cu^{2+} ions wherein most other fibrinolytic enzymes are inhibited in its presence (**Mukherjee & Rai, 2011; Xin *et al*., 2018**). Hence, given the similarity, the fibrinolytic enzyme isolated from *Bacillus toyonensis* too could be a Serine protease. The studies on the enzyme at various temperatures indicate that it is quite stable in a wide range of temperatures. This result is similar to the proteases obtained from other Bacillus species such as, *Bacillus pseudofirmus* SVB1, which exhibited equally high stability and activity at 40 °C (**Vijayaraghavan *et al*., 2015**). Since EDTA did not have any effect on the enzymatic properties, it could indicate that the purified enzyme is not a metalloprotease. Though, PMSF, a known serine protease inhibitor did not inhibit the purified enzyme, it cannot be ruled out that our enzyme is not a serine protease. It could indicate that the enzyme has a greater chemical endurance ability than other serine proteases and can remain active under broad conditions. The reason for this enhanced activity could be that surfactants like SDS lead to folding of the enzyme structure into compact barrel motifs, which in turn maximizes the enzyme activity (**Taneja *et al*., 2017**).

The optimal temperature of the enzyme activity is close to the physiological temperature and would hence be suitable for use in thrombolytic therapy. Its pharmaceutical importance was further enhanced by its pronounced stability in the presence of various organic agents as well as metal ions.

CONCLUSION

In conclusion, the results of the various biochemical tests performed on the enzyme indicate that it can have a great potential to be further developed as a thrombolytic agent as it has been shown to have sustained high fibrinolytic activity. Further studies on the physiological functioning of the purified enzyme and the capability for in-vivo lysis of thrombi can be carried out in the future. Hence, endophytic bacteria present a hitherto largely unexplored avenue for future research and discovery of novel fibrinolytic enzymatic agents to treat thrombosis.

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