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Research Article

A Novel Fibrinolytic Enzyme from *Bacillus Sphaericus* MTCC 3672: Optimization and Purification Studies

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Abstract

A novel extracellular fibrinolytic enzyme was produced from *Bacillus sphaericus* MTCC 3672 for dissolving blood clots. Optimized fermentation parameters achieved by one factor at a time approach had demonstrated 2.85 fold increase in fibrinolytic activity i.e. from $3.5*10^4$ U/l (basal media) to $9.98*10^4$ U/l after 24 h of incubation in submerged fermentation. Statistical screening of six independent nutritional variables such as, glucose, yeast extract, NaCl, $MgCl_2$, $MnCl_2$, $CaCl_2$ was studied using Plackett-Burman design. Amongst six variables, yeast extract was found to be significant factor affecting yield of a fibrinolytic enzyme. Furthermore, growth kinetics of biomass formation, enzyme production, and substrate utilization was evaluated by unstructured kinetic models and various biokinetic parameters such as μ_{max} (0.37 1/h), P_r (0.12 1/h), $Y_{P/S}$ (7.74 U/g) and $Y_{X/S}$ (3.32 g/g) were determined. In purification step, ultrafiltered broth was purified with DEAE Cellulose anion exchange chromatography and Sephadex G100 gel filtration chromatography with 10 fold purity. *In vitro* fibrin clot degradation study had revealed significant breakdown of fibrin clot. The fibrinolytic activity of purified enzyme (7.5 mm) was found to be matching with marketed Nattolife® (8.0 mm) (nattokinase) and superior to Thromboflux® (0 mm) (streptokinase) formulations. Hence, efficient formulation containing purified fibrinolytic agent can be used for medical emergencies viz. myocardial infarction and deep vein thrombosis.

Keywords: Fibrinolytic enzyme; Bacillus sphaericus; Plackett-Burman design; Kinetic Models; DEAE Cellulose Sephadex G 100

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Introduction

Intravascular thrombosis, formation of a blood clot in a blood vessel, is one of the main causes of cardiovascular diseases (CVDs). Fibrinolytic agents are promising and highly effective therapy for CVDs, classified in two groups as a plasminogen activators and plasmin like protein type fibrinolytic enzyme [1]. Plasminogen activator converts plasminogen to plasmin, which eventually degrades fibrin clot to fibrin degraded product. Streptokinase (EC 3.2.1.35), tissue plasminogen activator (t-PA), urokinase (u-PA, EC 3.4.21.31) and reteplase are commercially available plasminogen activators, which experiences various drawback such as requirement of large therapeutic dose, short plasma half-life, limited fibrin specificity, antigenic reactions, reocclusion and bleeding complications [2]. On the other hand, plasmin like protein type fibrinolytic enzyme such as nattokinase, lumbrokinase (EC 3.4.17.13) and fibrolase, directly degrades fibrin framework of thrombi, and dissolves blood clot rapidly and completely [3]. Directly acting fibrinolytic enzyme is found to be superior therapy for treatment of CVDs. Hence, practically there is need of a novel and highly potent fibrinolytic agent which can directly degrades fibrin clot in lesser time with improved specificity and overcome shortcoming of hemorrhage.

Various fibrinolytic enzyme are derived from different plant, animal and microbial sources. Amongst various sources, microbial fibrinolytic agents have attracted attention of research community due to advancement in molecular biology and fermentation technology.

Bacillus genus is a well known producer of a potent fibrinolytic enzyme such as nattokinase (*Bacillus natto)* and Subtilisin DEF (*Bacillus Amyloliquefeficiens)* [4, 5]. However, other *Bacillus* species e.g., *Bacillus sphaericus* have been established in market as a potent insecticide. *Bacillus sphaericus* H5a5b serotype produces intracellular toxin for mosquito larvae. Accidentally, it had shown fibrinolytic activity in extracellular extract [6-8]. Yet, to the best of our knowledge, no one has reported evaluation and production of fibrinolytic enzyme from *Bacillus sphaericus* MTCC 3672.

Conventionally, fermentation was optimized by one factor at a time approach of medium optimization, illustrating the effect of each variable individually on response. However, statistical approach signifies mutual interaction in between independent variables and estimates accurate value of dependent variable in minimum time with reduced experimentation. Plackett Burman design [9] is one of such widely used statistical tools for screening of large number of nutritional parameters. In addition to this, kinetic modeling of fermentative growth by various unstructured model is very important for precise understanding of production profile of enzymes.

Purity of therapeutic enzyme is a prime concern to be monitored during bioprocesses. Generally, purification protocol incurred 50-80% cost of therapeutic enzyme manufacturing. So, fermentation broth should be processed efficiently to achieve maximum purity in minimum no. of steps at economically viable cost.

Need of directly acting fibrinolytic enzyme with higher purity laid the foundation for our research work. The objective of the present investigation was aimed to produce, optimize, purify and evaluate a novel fibrinolytic enzyme from *Bacillus sphaericus* MTCC 3672 (wild type strain) by combination of one-factor-at-a-time and statistical approach of medium optimization. The growth kinetics was studied with unstructured models. Fibrinolytic activity of purified enzyme was evaluated.

Materials and Method

Chemical and reagents

All analytical grade chemicals and media components were purchased from S. D. Fine-Chem Ltd., Mumbai, India and Hi-Media Ltd., Mumbai, India, respectively. Azoalbumin was purchased from Sigma Aldrich, India. Nattolife® and Thromboflux® were provided as a gift sample from Zytex India Pvt. Ltd., Mumbai, India and Bharat Serum and Vaccines, Mumbai, India, respectively.

Microorganism and its maintenance

Bacillus sphaericus MTCC 3672 was obtained from Microbial Type Culture Collection and Gene bank, Chandigarh, India and was maintained on agar slants of medium containing (g/l) peptone, 5; NaCl, 15; beef extract, 1; yeast extract, 2 and agar 20; (pH 7.0) at 37 °C for 1 day and then stored at 4 °C.

Analytical Method

Measurement of protease activity

Proteolytic enzyme content was determined by Li and Yousten [10] method using 0.5% (w/v) of azoalbumin in 0.1 M morpholino propane sulfonic acid (pH 7.0) as a substrate. The assay mixture contains 0.3 mL of enzyme, 1 mL of 0.5% (w/v) azoalbumin in 0.1 M morpholino propane sulfonic acid (pH 7.0) and 0.7 mL of DI water. It was incubated at 30 ºC for 1 h. 2 mL of 8% (w/v) trichloro acetic acid reagent was added to above assay mixture for precipitation and resultant precipitate was removed by centrifugation at 10000 rpm for 15 min. 2 mL of supernatant was added to 2 mL of 0.5 M NaOH and absorbance was measured at λ_{max} 440 nm (Spectroscan UV 2700, Double Beam UV-Vis Spectrophotometer, Chemito, India). One unit of enzyme activity was considered as that amount of enzyme which produced an absorbance increase of 0.01, under the assay conditions of pH 7.0, temperature 30 °C and incubation time of 1 h.

Measurement of protein content

Protein concentration was determined by Bradford method using Coomassie Blue G250 [11].

Dry cell weight determination

Dry cell weight (DCW) was measured by centrifuging 2 mL of culture broth in a preweighed microcentrifuge tube at 10,000 g for 5 min. Supernatant was decanted and cell pellet was washed twice with DI water, and again centrifuged. Cell pellet was dried at 80 °C for 24 h.

Fermentation

Fermentation experiments were carried out in an Erlenmeyer flask (250 mL) containing 50 mL of production media (% w/v) [12] composed of glucose, 1; peptone, 0.5; NaCl, 0.5; beef extract, 0.3; yeast extract, 0.05; $MgCl_2$, 0.0203; $CaCl_2$, 0.01; $MnCl_2$, 0.001; pH, 7.0. Sterilized media was inoculated with 2 mL cell suspension (approximately $2.7*10^8$ cells/mL with 0.7 OD @ 600 nm) of 1 day old culture suspended in saline solution and incubated at 37 °C for 24 h at 180 rpm (Slant age, inoculum volume were optimized experimentally. Data was not shown).

Optimization studies of fermentation medium using one-factor-at-a-time method

Effect of various process variables was determined using one factor at a time method. In order to investigate effect of initial media pH and

temperature on production of fibrinolytic enzyme, medium with different initial pH (5.0-9.0 at temperature 37 °C) and different temperature (25, 30, 37 and 40 °C at pH 8) were incubated for 24 h with *Bacillus sphaericus* MTCC 3672. Protease activity and DCW were determined.

Different carbon, nitrogen, salt ion sources were screened for evaluating its effect on production of enzyme. Protease activity and DCW were calculated after 24 h of incubation at 30 ^{0}C temperature and pH 8.

Screening of most significant nutrients components by Plackett-Burman Design

Plackett-Burman design [9] was used to determine most significant nutritional components affecting production of a fibrinolytic enzyme. Seven (n) variables including six nutritional factor such as concentration of glucose, yeast extract, NaCl, $MgCl_2$, $CaCl_2$, $MnCl_2$ and one dummy variable were screened in eight (n+1) experiments (Table 2).

The each variables was examined at high (+) and low (-) level of concentration, these concentration were selected from earlier medium optimization study. The total no. of (+) and (–) signs per column should be equal.

The effect of each variable was determined by equation shown as Eq. (1).

$$E_{(X_i)} = \frac{2(\sum P_{i+} - \sum P_{i-})}{N} \quad (1)$$

where, $E_{(Xi)}$ is concentration effect of tested variable, and P_{i+} and P_{i-} represents enzyme production from the trials where the variables (X_i) being measured were added to the production medium at high (+) and low (-) concentrations, respectively, and N is the number of experiments performed.

Kinetic Modeling

After screening of significant factors affecting production of fibrinolytic enzyme, understanding of key production parameters at industrial level is necessary. Various structured and unstructured models are available in the literature for estimation of the biokinetic parameters of fermentation and for understanding of production pattern. Some of unstructured models such as, Monod kinetic model, logistic equation and Haldane model are widely used owing to its simplicity. Logistic equation is substrate independent model which precisely describes the inhibition of biomass on growth. The logistic equation can be described as follows in Eq. 2:

$$X = \frac{X_0 X_{max} e^{\mu_{max} t}}{X_{max} + X_0 - X_0 e^{\mu_{max} t}} \quad (2)$$

where, t is time (h), X is biomass concentration (g/L), X_{max} is maximum concentration of biomass (g/L), and μ_{max} (h 1/L) is the ratio between initial volumetric rate of biomass formation and initial biomass concentration X_0 (g/L). The model parameters X_0, X_{max}, and μ_{max} can be calculated from series of experimental data of biomass concentration/time.

Fibrinolytic enzyme production was mathematically modeled using the equation proposed by Mercier *et al*. [13] for lactic acid fermentation as shown in Eq. (3):

$$p = \frac{p_0 p_{max} e^{p_r t}}{p_{max} + p_0 - p_0 e^{p_r t}} \quad (3)$$

Where, t is time (h), P is fibrinolytic enzyme activity (10^4*U/L), P_{max} is maximum fibrinolytic enzyme activity (10^4*U/L), and P_r is ratio between initial volumetric rate of product formation (r_p) and initial product concentration P_0 (10^4*U/L).

The substrate utilization kinetics can be interpreted by following expression shown in Eq.

(4) [14].

$$S = S_0 - \frac{1}{Y_{P/S}}(P - P_0) + \frac{1}{Y_{X/S}}(X - X_0) \quad (4)$$

where, $Y_{P/S}$ (U/g) and $Y_{X/S}$ (g/g) are product yield for fibrinolytic enzyme and biomass, respectively, P and P_0 is final and initial fibrinolytic enzyme activity (10^4*U/L), X and X_0 are final and initial biomass concentrations (g/L), and S_0 is initial substrate concentration (g/100 mL).

Statistical Analysis

One way ANOVA was used for statistical analysis of results. P-value less than 0.05 was considered to be statistically significant. Graph pad prism 5.0 software (USA) was used for statistical analysis of data.

Enzyme Purification

After 24 h of incubation, cell broth was centrifuged at 10,000 g at 4 °C for 15 min and resultant supernatant was microfiltered. Filtered broth was concentrated using AMICON stirred cell ultrafiltration unit equipped with polysulphone membrane of 10 Kd MWCO. Nitrogen gas was maintained at 2 bar pressure and retentate was separated and dialysed against 10 mM Tris-HCl buffer, pH 8.0 overnight.

The dialysate was applied to 5 cm DEAE-Cellulose column equilibrated with 10 mM Tris-HCl buffer, pH 8.0 at 30 °C. After washing, column was eluted with 0.5 M NaCl in 10 mM Tris-HCl buffer, pH 8.0 with 0.5 mL/min flow rate. Each fraction of 2 mL was collected and enzymatic activity exhibiting fractions were pooled together and dialysed against 10 mM Tris-HCl buffer, pH 8.0 at 4 °C. Dialysed sample was loaded on Sephadex G 100 column and eluted with 0.5 M NaCl in phosphate buffer saline pH 7.4 at a flow rate of 0.5 mL/min. Fractions of 1 mL were collected. Each fraction was monitored at 280 nm for protein and assayed for enzymatic activity.

In Vitro blood clot degradation

In vitro fibrinolytic activity was determined by artificial blood clot degradation method. Blood clot was formed by spontaneous coagulation of freshly collected human blood in a glass tube. After 1 h, artificial blood clot was rinsed out thoroughly and dipped in 10 mL of phosphate-buffered saline (pH 7.0) containing purified fibrinolytic enzyme and normal saline as a control at room temperature for clot dissolution [15].

Fibrin plate assay

Fibrin plate assay was performed to determine and compare fibrinolytic activity of purified enzyme with commercially available fibrinolytic agent such as Nattolife® (nattokinase) and Thromboflux® (streptokinase).by reported method with some modification [16]. Thefibrin agarose plates were formed using 2% w/v agarose, 0.6% w/v fibrinogen, and 20 NIH/mL of thrombin. The fibrin clot was allowed to stand for 1 h at room temperature. 40 µl of enzyme solution, 600 IU of Thromboflux® and 1.2 FU of Nattolife® were inoculated onto the wells of plate and incubated for 12 h at 37 ^{0}C and diameter of lytic circle was measured.

Result and discussion

Optimization of fermentation medium using one-factor-at-a-time method

Effect of initial medium pH on production of fibrinolytic enzyme was demonstrated in **Table 1**. Optimum pH for maximum production of fibrinolytic enzyme was found to be 8.0 and total protease activity was $5.27*10^4$ U/L. At optimum pH, there was maximum gene expression of fibrinolytic enzyme and higher biomass formation. Hence, all further fermentation studies were performed at pH 8.0. Most of the *Bacillus* spp. grows best in a neutral or slightly basic pH environment. Mahadevan *et al*. [7] had studied production of thrombinase at pH 7.4 form *Bacillus sphaericus* serotype H5a5b.

Enzyme production at various temperatures was shown in **Table 1**. At 30 °C, *Bacillus sphaericus* MTCC 3672 culture had shown higher enzyme activity ($5.47*10^4$ U/L). However, higher temperature yielded lower enzyme production; this may be due to change in physiological activities. *Bacillus* spp. shows prominent growth in the temperature range of 30-37 ^{0}C; such as *B. subtilis* had produced fibrinolytic enzyme at 37 ^{0}C [17]. Our result complies with protease producing *Bacillus spp*.

Nitrogen regulation is the significant factor of industrial microbiology as it affects synthesis of various enzymes involved in both primary and secondary metabolism of microorganism. Many secondary metabolic pathways are negatively affected by nitrogen sources. Researchers have reported that bacteria use reduced form of nitrogen source for biosynthesis of enzyme. After screening of different organic and inorganic nitrogen sources; beef extract, peptone, and yeast extract have shown higher enzyme activity such as $3.3*10^4$ (U/l), $3.52*10^4$ (U/l), and $5.5*10^4$ (U/l), respectively (**Table 1**). Organic nitrogen sources have inducing effect on protease production. Earlier, researchers have developed cost effective media from different organic nitrogen sources such as, soybean flour and egg yolk powder for maximum growth of *Bacillus sphaericus* H5a5b [8, 18]. On the other hand, inorganic nitrogen sources had shown low bacterial growth and lower enzyme production because of its repressor effect. Optimization of yeast extract concentration had shown higher enzymatic activity at 1.5% w/v yeast extract concentration and inhibitory effect at higher concentration (**Table 1**). Similar observation was reported by Cho *et al*. for decreased cell growth and reduced nattokinase production from *Bacillus subtilis* at higher supplementation of yeast extract [19]. The varying fibrinolytic enzyme activity with respect to yeast extract concentration demonstrates its vital significance as a media component.

The major and minor salt catalyzes various microbial metabolic pathways responsible for production of secondary metabolites. The basal media contains NaCl, $CaCl_2$, $MgCl_2$ and $MnCl_2$ as a salt ion source. The substitutes for each ion were tested and effect of each salt on biomass production and enzyme activity was determined and shown in **Fig. 1.** From figure, it was clear that basal media salt yielded maximum yield as compared to substituted salts.

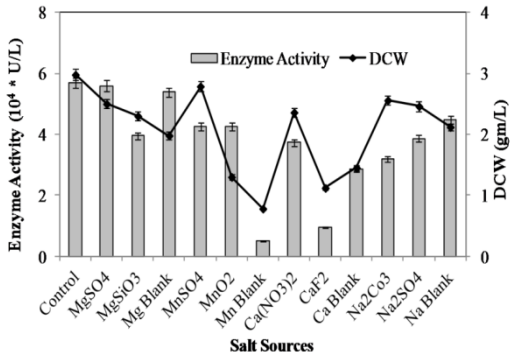

Figure 1 Salt ion optimization for *Bacillus sphaericus* MTCC 3672 production. Enzyme activity and DCW was determined after 24 h of incubation at pH 8, temperature 30 ºC.

In microbial fermentation, carbon source not only acts as a major constituent of cellular membrane, but also act as an energy source for various activities. Amongst all carbon sources, glucose showed highest enzyme production (**Table 1**). Earlier researchers have proved that glucose is a preferred carbon source for growth of *Bacillus spp*. Minimum solubility, poor cellular uptake and unsuitable metabolic pathway of other carbon sources resulted in reduced production of fibrinolytic enzyme. **Table 1** demonstrated optimum concentration of glucose (1.25% w/v) for maximum enzyme activity. However, different glucose concentration (0-2.5% w/v) had shown slight variation in fibrinolytic enzyme production, suggesting its non production limiting nature. Similar findings were reported by Prabakaran *et al*, *Bacillus sphaericus* doesn't require carbon source for their growth or it grows very poorly in presence of glucose, its growth totally depend on proteinaceous material [8]. Our result also re-signifies earlier researcher's findings. The absence or presence of glucose source doesn't have any significant effect on production of fibrinolytic enzyme from *Bacillus sphaericus* MTCC 3672. The optimized medium had significantly increased yield of fibrinolytic enzyme from $3.5*10^4$ (U/l) to $9.98*10^4$ (U/l) by one-factor-at-a time method ($P<0.05$).

Screening of most significant nutrients components by Plackett-Burman Design

Plackett-Burman design was employed for determination of significant nutritional components and shown in **Table 2** along with results of experimental trials and ANNOVA analysis. Higher value of effect either positive or negative indicated its crucial impact on yield, while effect value closer to zero revealed that factor had little or no effect on yield. In **Table 2**, yeast extract had shown higher negative effect value indicating its significance for fibrinolytic enzyme production but amount required was lower than stated low (-) level of concentration of yeast extract in Plackett-Burman design.

The mean square value and F test value for yeast extract were observed to be higher than other nutritional and dummy variable, which clearly signified that yeast extract was the sole important nutritional component affecting production of fibrinolytic enzyme. Fibrinolytic enzyme yield was increased from $3.5*10^4$ U/l to $11.1*10^4$ U/l (3.12 fold) after Plackett-Burman design.

Kinetic Modeling

The widely known logistic equation was fitted to represent batch fermentation kinetics of *Bacillus sphaericus* MTCC 3672. The experimental data was fitted into the model equation and resulted biokinetic parameters were shown in **Table 3**.

Experimental and model value of biomass depicted in **Fig.2A** revealed that, lag phase of microbial growth was lasted upto 4 h during which microorganism tried to get acclimatized to the new environment and prepared themselves for cell multiplication. Exponential phase was started from 4 h and last upto 24 h of incubation, after which stationary phase was started due to depletion in nutrients supplement. Maximum biomass growth obtained at 24 h was 4.56 gm/l and it was gradually decreased till 48 h. Many proteases have shown maximum production in stationary phase of growth curve.

Table 1 One-factor-at-a-time optimization of production of fibrinolytic enzyme from *Bacillus sphaericus* MTCC 3672

[a, b] Enzyme activity and DCW after 24 h of incubation of 2 mL of cell suspension (approximately $2.7*10^8$ cells/mL) of 1 day old culture of *Bacillus sphaericus* MTCC 3672; [c] Results are mean SD of at least 3 determination.

pH	**5**	**6**	**6.5**	**7**	**7.5**	**8**	**9**
[a]Enzyme Activity (U/L)*10^{4c}	4.17±0.2	4.93±0.28	5.35±0.3	5.37±0.29	5.47±0.21	6.27±0.26	4.40±0.16
[b]DCW (gm/L)[c]	0.43±0.01	0.72±0.02	1.1±0.05	1.3±0.03	2.19±0.04	2.51±0.05	2.2±0.0.6
Temperature °C	**25**	**30**	**35**	**40**			
Enzyme Activity (U/L)*10^4	3.72±0.12	5.47±0.19	2.43±0.09	2.52±0.11			
DCW (gm/L)	1.3±0.05	2.83±0.08	2.41±0.08	2.06±0.08			
Organic Nitrogen (1% w/v)	**Peptone**	**Yeast Extract**	**Beef Extract**	**Skimmed Milk**	**Soya Peptone**	**Soya Grits**	**Casein**
Enzyme Activity (U/L)*10^4	3.52±0.11	5.50±0.21	3.30±0.12	2.00±0.15	2.37±0.10	0.90±0.01	0.52±0.01
DCW (gm/L)	2.53±0.09	3.61±0.12	2.62±0.12	2.14±0.05	1.98±0.04	0.78±0.01	0.65±0.01
Inorganic Nitrogen (1% w/v)	**$NaNO_3$**	**$Ca(NO_3)_2$**	**NH_4NO_3**	**$(NH_4)_2S0_4$**	**NH_4Cl**		
Enzyme Activity (U/L)*10^4	0.97±0.03	0.47±0.01	0.32±0.01	0.70±0.02	0.33±0.01		
DCW (gm/L)	0.45±0.02	0.31±0.01	0.29±0.01	0.49±0.02	0.23±0.01		
Yeast Extract concentration (% w/v)	**0**	**0.5**	**0.75**	**1**	**1.5**	**2**	**3**
Enzyme Activity (U/L)*10^4	0.55±0.21	3.60±0.12	4.80±0.18	5.55±0.20	5.63±0.22	3.30±0.12	1.43±0.05
DCW (gm/L)	0.12±0.01	1.6±0.01	2.67±0.08	2.92±0.10	3.67±0.14	2.84±0.10	2.35±0.09
Carbon Source (1% w/v)	**Glucose**	**Fructose**	**Maltose**	**Lactose**	**Sucrose**	**Glycerol**	
Enzyme Activity (U/L)*10^4	6.3±0.29	5.05±0.20	4.42±0.19	4.50±0.20	3.93±0.12	1.22±0.05	
DCW (gm/L)	4.03±0.15	3.32±0.10	2.63±0.11	2.45±0.09	2.13±0.06	1.5±0.02	
Glucose concentration(% w/v)	**0**	**0.5**	**1**	**1.25**	**1.5**	**2**	**3**
Enzyme Activity (U/L)*10^4	7.42±0.30	8.52±0.39	8.93±0.41	9.86±0.42	8.64±0.38	8.18±0.32	4.92±0.21
DCW (gm/L)	3.6±0.12	3.97±0.13	4.12±0.15	4.41±0.15	4.19±0.12	3.81±0.11	3.6±0.12

Table 2 Plackett-Burman design and ANNOVA analysis for screening of nutrient parameters affecting fibrinolytic enzyme production

Run	Glucose	Yeast extract	NaCl	$MgCl_2$	$CaCl_2$	$MnCl_2$	Dummy	U/mL[a]
1	(+)	(+)	(+)	(-)	(+)	(-)	(-)	35.67±0.64
2	(-)	(+)	(+)	(+)	(-)	(+)	(-)	41.33±0.91
3	(-)	(-)	(+)	(+)	(+)	(-)	(+)	93.00±1.18
4	(+)	(-)	(-)	(+)	(+)	(+)	(-)	91.33±0.95
5	(-)	(+)	(-)	(-)	(+)	(+)	(+)	57.67±1.09
6	(+)	(-)	(+)	(-)	(-)	(+)	(+)	111.0±1.29
7	(+)	(+)	(-)	(+)	(-)	(-)	(+)	44.33±0.61
8	(-)	(-)	(-)	(-)	(-)	(-)	(-)	30.00±0.75
∑(H)	282.33	179	281	270	277.67	301.33	306	
∑(L)	222	325.33	223.33	234.33	226.67	203	198.33	
Difference	60.33	-146.33	57.67	35.67	51	98.33	107.67	
Effect	15.08	-36.58	14.42	8.92	12.75	24.58	26.92	
Mean Square	455.01	2676.68	415.68	159.01	325.12	1208.68	1449.01	
F test	0.31	1.85	0.29	0.11	0.22	0.83	1	

Glucose high level (+) = 1.5gm, low level (-) = 1.0 gm; Yeast extract (+) =2.0 gm, (-) = 1.0 gm; NaCl (+) = 1.0 gm, (-) = 0.2 gm; $MgCl_2$ (+) = 80 mg, (-) =20 mg; $CaCl_2$ (+) = 20 mg, (-) = 4 mg; $MnCl_2$ (+) = 4 mg, (-) = 1.0 mg.

[a] Results are mean ± SD of three determinations.

Table 3 Values of the biokinetic parameters for biomass formation, product formation, and substrate utilization by *B. sphaericus* MTCC 3672 batch fermentation

Biomass			**Product**			**Substrate**		
X_0 (g/L)	X_{max} (g/L)	μ_{max} (1/hr)	P_0 (U/L*10^4)	P_{max} (U/L*10^4)	P_r (1/hr)	S_0 (g/L)	$Y_{P/S}$ (U/g)	$Y_{X/S}$ (g/g)
0.62	5.01	0.37	6.72	10.50	0.12	1.25	7.74	3.32

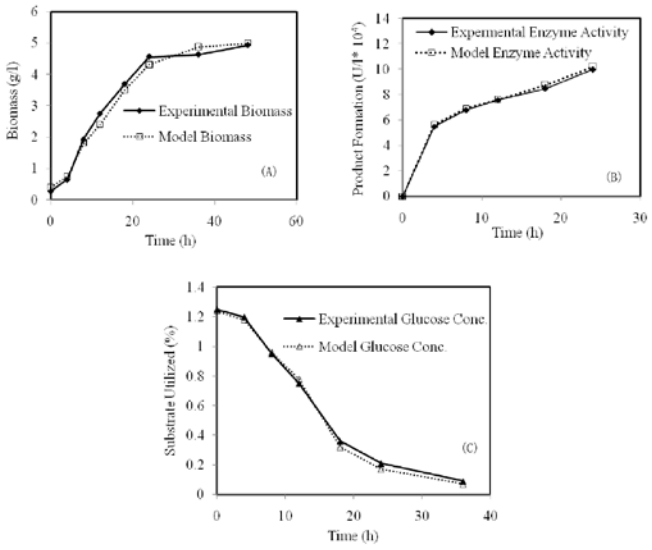

Figure 2 Comparison of experimental and model values of (A) biomass formation (g/L), (B) product formation (U/L * 10^4) and (C) substrate utilized (w/v %) with respect to time.

The model equation proposed by Mercier *et al.*, was used for evaluating product formation kinetics (**Fig. 2B**). The enzyme production was gradually increased along with exponential phase and attained maxima (in 24 h to 9.98 *10^4 U/L) in stationary phase and remained constant thereafter before entering to death phase. The production of fibrinolytic enzyme ceased after stationary phase, it may be due to autoproteolysis or degradation of protease by some other protease produced aside in fermentation broth. *Bacillus sphaericus* MTCC 3672 had signified non-growth associated production of fibrinolytic enzyme; similar to nattokinase production from *Bacillus natto* NRRL 3666 [15].

Substrate concentration was gradually decreased from 1.25% to 0.09% (w/v) with enhanced biomass and product formation (**Fig. 2C**). Maximum glucose supplement was used for cell multiplication and energy generation. S_0 was calculated by fitting experimental data of substrate utilization in Eq. 4.

The least square method of non linear regression was used to calculate biokinetic parameters of biomass, product formation and substrate utilization (μ_{max}, X_0, X_m; P_0, P_r, Pmax; S_0, $Y_{P/S}$, and $Y_{X/S}$). **Table 3** summarized all constants and correlation regression coefficients obtained for all the models by statistical computing. Fig. 2A, 2B, and 2C shows comparison of experimental and model predicted values for shake flask level biomass growth (R^2=0.9745), enzyme production (R^2 =0.9857), and substrate utilization (R^2 =0.9912), respectively. Close resemblance of experimental and model values had validated the proposed models.

Enzyme Purification

The microfiltered (0.22 µm) crude having 202 U/mg of specific activity was purified by 3 step purification protocol. It was concentrated by ultrafiltration using polysulphone membrane of 10 kDa MWCO (Amicon, Inc., U.S.A.) to 1.49 fold purity with 58% yield. Dialysate of ultrafiltration retentate was purified by DEAE Cellulose anion exchange chromatography **(Fig 3A)**. The pooled protein fractions had shown

6.42-fold purity with 60% enzyme recovery after elution with 0.5 M NaCl in 10 mM Tris HCl, pH 8.0 buffer. Active fractions were pooled together and dialyzed against10 mM Tris HCl, pH 8.0 buffer. Pooled fractions were further purified by Sephadex G-100 gel chromatography. Elution profiles of protein and proteolytic activity were shown in **(Fig. 3B)**. The final purified extracellular fibrinolytic enzyme had shown 10 fold purity after gel filtration chromatography. Protease from *Aspergillus clavatus* ES1 strain was purified using acetone precipitation, Sephadex G-100 gel filtration and CM-Sepharose ion exchange chromatography, with a 7.5-fold increase in specific activity and 29% recovery [20].

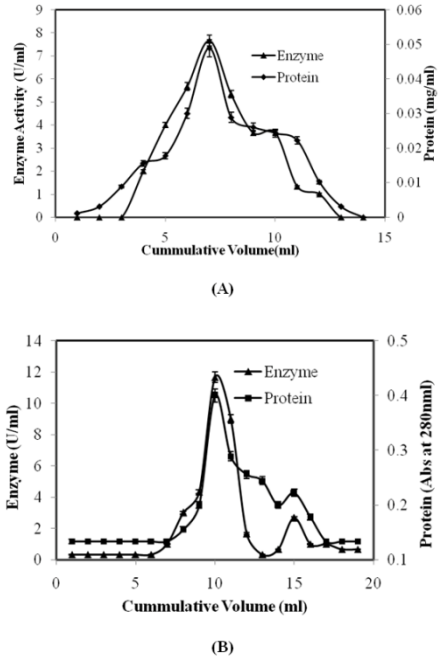

Figure 3 Enzyme elution profile of (A) DEAE Cellulose anion exchange chromatography, (B) Sephadex G100 gel filtration chromatography

In vitro blood clot degradation

In vitro blood clot degradation was performed using enzyme of *B. sphaericus* MTCC 3672 and 0.9% NaCl saline as a control. Purified enzyme had shown significant degradation of blood clot, while control was devoid of any degradation after 1 h as shown in **Fig.4A**. Hence, *In vitro* blood degradation study had successfully demonstrated that, produced enzyme possesses a fibrinolytic activity. Mahajan *et al*, has demonstrated similar *in vitro* blood clot degradation by nattokinase produced from *B. natto* NRRL 3666 [15].

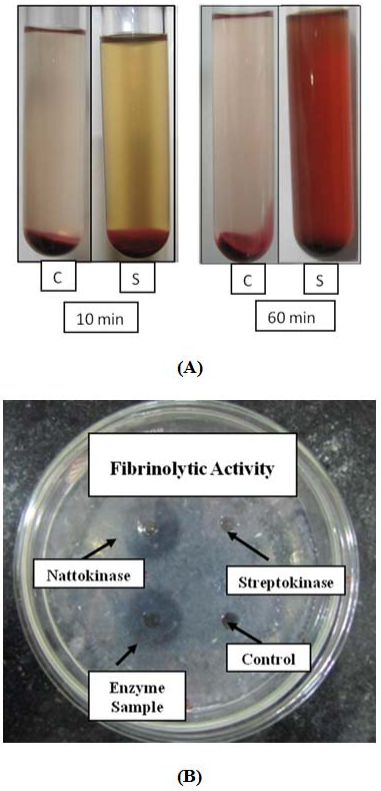

Figure 4 (A) Degradation of artificial blood clot by fibrinolytic enzyme from *Bacillus sphaericus* MTCC 3672 after 1 h incubation. S, blood clot with enzyme solution; C, blood clot with saline solution, (B) Fibrinolytic activity determination by fibrin plate assay; Incubation of plate at 37 ºC for 1 h.

Fibrin plate assay

Comparison of fibrinolytic activity of purified enzyme with Nattolife® and Thromboflux® was shown in **Fig. 4B**. The fibrin agarose plate

was devoid of plasminogen. The zone of fibrin lysis of produced enzyme (7.5 mm) and Nattolife® were almost similar (around 8 mm), whereas Thromboflux® (0 mm) was unable to clear the fibrin clot. Results of fibrinolytic activity emphasized that produced enzyme directly acts on fibrin clot and thus can be categorized as a plasmin like protein type directly acting fibrinolytic enzyme. Nattokinase had shown similar mode of action in plasminogen devoid plate [21]. Hence, a novel directly acting fibrinolytic enzyme has almost similar activity to nattokinase and is plamin like protein.

Conclusion

The present investigation has highlighted production, purification and evaluation of a novel fibrinolytic enzyme from *Bacillus sphaericus* MTCC 3672 by submerged aerobic fermentation. The optimized media given by one-factor-at-a-time method had produced $9.98*10^4$ (U/l) yield. Plackett-Burman design confirmed the importance of yeast extract as significant medium component and proved as an efficient screening tool. Unstructured kinetic models were validated for different responses. Higher potency and direct fibrinolytic action of a novel enzyme was evaluated by fibrin plate assay and *in-vitro* blood clot degradation. Hence, an optimized medium and purification protocol established through present investigation will be useful for the development of an alternative to conventional fibrinolytic agents for cardiovascular disease treatments. Extensive characterization and formulation development are on track.

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