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Original article

In vitro thrombolytic potential of fibrinolytic enzyme from *Brevibacterium* sp. isolated from the root of the plant, *Aloe castellorum*

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ABSTRACT

Cardiovascular diseases (CVDs) like stroke and heart attack are leading causes of mortality worldwide, resulting in about 30% of deaths. The proportion in the Kingdom of Saudi Arabia was 46%. The commercial thrombolytic agents are thus associated with a range of side effects, while bacterially derived fibrinolytic enzymes have no or little side effects. Hence, there is an urgent need to identify novel fibrinolytic enzymes to treat or prevent CVDs. An endophytic bacterial strain producing fibrinolytic enzyme was isolated from the root of the plant *Aloe castellorum*. Enzyme production was enhanced through a conventional method and a statistical approach. The in vitro lytic activity of blood clots was also assessed. Endangered plant of *Aloe castellorum* roots, researchers isolated an endophytic strain producing fibrinolytic enzymes. It was identified as *Brevibacterium sp*. The culture medium was also optimized by a conventional method to screen the factors significant for successful culture. Fibrinolytic enzyme production peaked when *Brevibacterium* sp. was cultured in the presence of maltose (613 ± 12 U/mL), followed by starch (576 ± 13 U/mL). Among the selected nitrogen sources, yeast extract (642 ± 5.9 U/mL) and beef extract (610 ± 13 U/mL) enhanced enzyme yield relative to that in the control (487 ± 7.2 U/mL). Ionic sources such as Mg^{2+} (672 ± 10.3 U/mL) and Ca^{2+} (605 ± 12.3 U/mL) showed enhanced fibrinolytic enzyme production relative to the control (405 ± 13.2 U/mL). Upon employing a two-level full factorial design for examining the significant factors, it was revealed that maltose, yeast extract, and Mg2+ were important ($p < 0.05$). To enhance thrombolytic agent production using central composite design and response surface methodologies. The fibrinolytic enzyme obtained from *Brevibacterium* sp. has great potential to lyse blood clots in vitro.

1. Introduction

Cardiovascular diseases (CVDs) cause more deaths worldwide than any other diseases. They cause about 30% of all deaths globally and 17.3 million people died of them in the year. Among the six Gulf Cooperation Council countries (GCC), CVDs were estimated to account for 45% of deaths, and according to the WHO, CVDs accounted for 46% of deaths in the Kingdom of Saudi Arabia (WHO, 2011). The overall number of deaths due to heart attack and stroke is expected to reach 23.3 million by 2002–2030 (Mathers et al., 2006).

Thrombosis occurs when blood clots appear in the blood vessels and is a major causes of Cardiovascular diseases, resulting in heart attack and thrombosis. Fibrin formed through the action of thrombin from fibrinogen is an important component of blood clots (Voet and Voet 1990). Naturally occurring plasminogen activator stimulates plasminogen and generates plasmin. This generated plasmin completely degrades insoluble fibrin (Dobrovolsky and Titaeva, 2002). The concept of fibrinolytic treatment involves the administration of plasminogen stimulator intravenously, which completely lyses thrombi that develop inside blood vessels and restores the bloodstream at sites of ischemia. Currently, three kinds of fibrinolytic agent are used for the treatment of CVDs: genetically engineered tissue plasminogen activator (t-PA), streptokinase, and

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urokinase. Though, these available thrombolytic agents are thermolabile, expensive and can cause number of side effects, especially susceptibility to allergic reactions and gastrointestinal bleeding (Turpie et al., 2002). Many fermented products have been used in recent years as sources for characterizing fibrinolytic enzymes, for example, *doenjang* (Kim and Choi, 2000), Korean *cheonggukjang* (Kim et al., 2009), skipjack *shiokara* (Sumi et al 1987), fermented rice (Vijayaraghavan et al., 2017; Vijayaraghavan et al., 2016) and douche (Peng et al., 2003). Bacillus subtilis in natto was shown to generate nattokinase as a fibrinolytic agent, Fibrin in blood clots is directly dissolved as well as t-PA is produced.

The t-PA produced activates plasminogen to active plasmin to dissolve fibrin. The *in vivo* administration of fibrinolytic enzymes was shown to enhance endogenous plasminogen activator in animals and humans (Kim et al., 1996). Fibrinolytic enzymes using microbes, mainly food-grade organisms, have the potential to be used as drugs or additive in health foods for the treatment or prevention of CVDs. Fibrinolytic enzyme-producing organisms were isolated from traditional fermented food and the isolated enzymes showed potent activity as well as fermented shrimp paste, these enzymes were characterized from as an nattokinase (Wong and Mine, 2004) which digested blood clots *in vivo* (Sumi et al., 1990). The aim of the present study is to fibrinolytic enzyme obtained from *Brevibacterium* sp. has great potential to lyse blood clots in vitro.

2. Materials and methods

2.1. Collection of plant root and isolation of bacteria

The roots of *Aloe castellorum* were extracted from the Al Baha Region in Saudi Arabia using sterile plastic containers and transported to the laboratory at 4 °C. The root samples were washed in running water, surface sterilized with 70% alcohol for 1 min, washed with 2.5% sodium hypochlorite solution for 4 min, rinsed three times in sterile distilled water and finally dried. For the isolation of bacteria, a previously reported procedure was used (Jain et al., 2021).

2.2. Screening of bacterial isolates for proteolytic activity

After the bacterial culture was purified, it was transferred to nutrient broth and incubated at 37 °C for 24 h in an orbital shaking incubator. A cell-free sample was then centrifuged at 10,000 × g for 10 min and the results then loaded onto skimmed milk agar plates that which were used to determine proteolytic activity. Proteolytic enzyme-producing organisms showed a clear halo zone around the well. The proteolytic enzyme-producing bacterial isolates were selected for secondary screening. (Vijayaraghavan et al., 2016)

2.3. Screening of bacteria for fibrinolytic enzyme activity

The method of fibrin agarose followed that of Astrup and Müllertz (1952). The fibrin agarose prepared as 0.75% (w / v) with agarose and 0.15% fibrinogen (Hiemedia, Mumbai, India) and used thrombin (0.125 ml of 200 NIH U / mL) (Sigma-Aldrich, USA) and the plate was left to stand for 1 h for complete fibrin clot formation. The wells at the center of the plates were also prepared by loading cell-free culture supernatant (20 μl). The fibrinolytic enzyme-producing organisms showed a clear zone around the well (Stephani et al., 2017).

2.4. Characterization of fibrinolytic enzyme-producing strain

The isolated bacterial strain producing the maximum level of fibrinolytic enzyme was selected for characterization. This strain was studied morphologically and biochemically (Dubey et al., 2013). DNA was extracted from the potent strain as defined by Yang et al. (2008). Analysis of the 16S rDNA gene of this candidate strain was executed universal forward (27 F, 5′-AGAGTTT GATCMTGGCTAG-3′) and reverse primers (R, 5′-ACGGGCGGTGTG TRC-3′).

2.5. Fibrinolytic enzyme test

The degradation of Fibrin was qualitatively determined by the method of (Stephani et al., 2017). Briefly, an artificial blood clot was prepared using thrombin (10 μl) on fibrinogen (100 μl) (Sigma Aldrich, USA). Then, 50 μl of the fibrinolytic enzyme was added to the substrate (fibrin) and incubated for 30 min. Next, trichloroacetic acid (TCA) (0.2 M) was added and the fibrin degradation was measured at 275 nm against a reagent blank using a UV–visible spectrophotometer. For standard assay conditions, the fibrinolytic enzyme activity of resembles to the quantity of enzyme required to release 1 M L-tyrosine per minute.

2.6. Inoculum preparation and submerged fermentation

An Erlenmeyer flask of 100 ml was used to administer nutrient broth medium and the bacterial strains (Himedia, Mumbai, India). In a rotary shaker incubator with a temperature of 37 °C and a rotation speed of 150 rpm, the culture was inoculated into the medium for 18 h. Double distilled water was used for the preparation of culture medium. Submerged fermentation was performed using a production medium (Kumar et al., 2018). The fermentation took place in a 250 ml Erlenmeyer flask with incubation at 37° C. for 2 days. After 48 h, the supernatant was separated with centrifuge for 15 min at 10,000 rpm and examined for enzyme activity.

2.7. Enhanced production of fibrinolytic enzymes by conventional method

Fibrinolytic enzyme production was affected by nutrient factors rather than environmental parameters in the case of submerged fermentation. For growth and enzyme production, carbon and nitrogen sources are both important factors in nutrient balance. Carbon sources such as starch, galactose, sucrose, lactose, maltose, and xylose were selected at the 1% (w/v) level. Nitrogen sources such as beef extract, casein, yeast extract, gelatin, peptone and ammonium sulfate were also included in an amount of 1% (w / v). The ions Ca2+, Mg2+, Mn+, Co2+, and Na+ were added to the production medium at the 0.1% (w/v) level. (Vijayaraghavan et al., 2016).

2.8. Optimization of fibrinolytic enzyme production by statistical method

In order to screen the variables that were most important for enzyme production, a two-level full factorial design was applied. Five variables (fermentation period, pH, yeast extract, maltose, and MgSO4) were selected for 32 experimental runs. In this experimental design, two levels (low and high) of values were selected and the yeast extract, maltose, and MgSO4 variables were found to enhance enzyme production. Therefore, these three variables were chosen to be further optimized. To analyze the optimal concentrations of these variables, central composite design (CCD) and response surface methodology were employed. CCD at five levels ($-\alpha$, $-$, 0, +, $+\alpha$) is described in Table 1. To design and validate

the experimental results, Design Expert statistical software was used.

Twenty experiments were performed for the selected three variables, which included six center points. The results were used to produce a 3D response surface plot and the optimal point was analyzed. All experiments were performed in triplicate, with the mean value being measured as the response (Y). ANOVA used to analyze the results. The experimental model was suited for second order the polynomial equation. (Vijayaraghavan et al., 2017).

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

Where a represents yeast extract, b represents maltose, and c represents $MgSO_4$.

2.9. Fibrinolytic enzymes as thrombolytic agents: In vitro analysis

The thrombolytic potential of fibrinolytic enzymes was analyzed via an in vitro experiment as described previously (Prasad et al., 2006). The blood clot was washed with PBS (pH 7.2) and the sample was added. The Streptokinase and buffer was used as positive and negative control. An hourly determination of the lytic activity of blood clot was carried out (Vijayaraghavan et al., 2016).

3. Results and discussion

3.1. Screening and characterization of fibrinolytic enzyme-producing bacteria

A root sample from a plant (*Aloe castellorum*) was used for the isolation of roots-associated endophytes. A total of around 17 bacterial isolates were obtained from the root sample and subjected to primary screening (protease screening). Out of these 17 bacterial isolates, the majority were found to be effective protease producers. Among the isolates, *Brevibacterium sp*. showed a 22 mm zone of inhibition around the bacterial colony on skim milk agar plates. From these protease-producing bacteria, the strain *Brevibacterium sp* hydrolyzed fibrin on fibrin-agarose plates (17 mm zone of hydrolysis). This strain was thus selected for characterization. The bacterium is gram-positive, not motile, nonspore-forming, and almost rod-shaped.

Its colonies are low-convex, circular with whole margins, methyl-red-positive, indole-negative and citrate utilization-positive. Molecular characterization was performed using 16S rDNA sequencing, as shown in Fig. 1a. The 16S rDNA sequence showed similarity to *Brevibacterium sp*. Generally, *Brevibacterium sp*. is an opportunistic pathogen and the proteases produced by it interact with the host during infection. This organism is involved in nosocomial and other clinical infections (Funke et al., 1997). *Brevibacterium* species have been identified in a variety of environments, including brown algae (Ivanova et al., 2004), moth caterpillars (Katı et al., 2010), and activated sludge (Kim et al., 2013). Recently, Renganath Rao et al. (2017) applied *Brevibacterium luteolum* (MTCC 5982) for the production of alkaline proteases for eco-friendly applications.

3.2. Improved fibrinolytic enzyme production by conventional method

Fibrinolytic enzyme production peaked when the *Brevibacterium* sp. was cultured in the presence of maltose (613 ± 12 U/mL), followed by starch (576 ± 13 U/mL) (Fig. 1b). Carbon sources

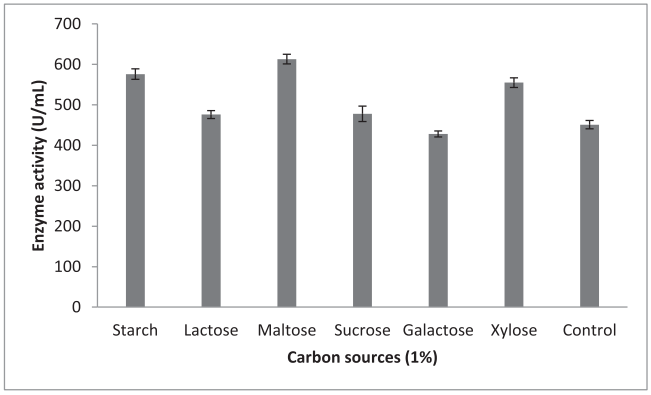

Fig. 1b. Production of fibrinolytic enzymes by carbon sources in submerged fermentation. About 1% carbon sources were incorporated into the production medium, which was incubated for 24 h. Error bar represents standard deviation.

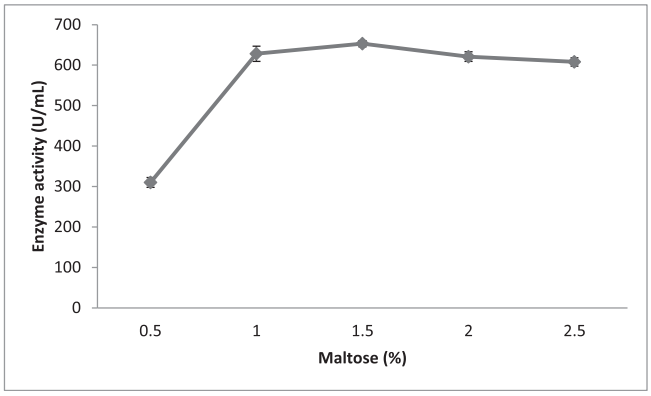

Fig. 1c. Effect of various concentrations of maltose on fibrinolytic enzyme production in submerged fermentation. Maltose was incorporated into the production medium, which was incubated for 24 h. Error bar represents standard deviation.

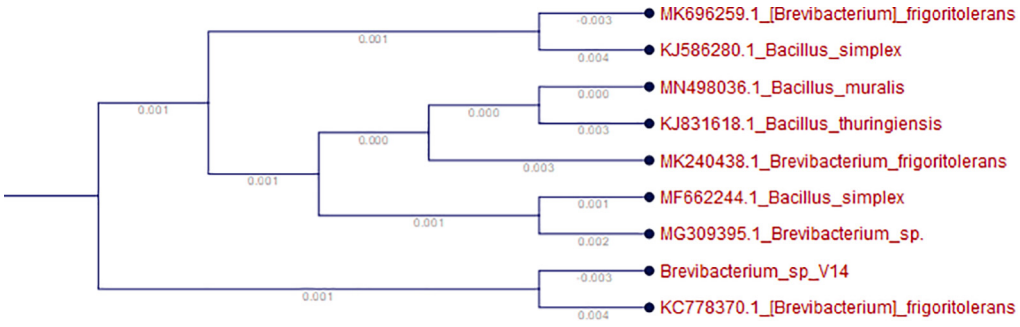

Fig. 1a. Phylogenetic assessment of 16S rDNA *Brevibacterium* sp. isolated from the root of a plant, *Aloe castellorum,* with other bacterial strains obtained by BLASTN. The sequences of the bacteria were aligned for the construction of a phylogenetic tree.

such as sucrose, galactose, and starch had the least influence on fibrinolytic enzyme production. The carbon sources in the culture medium influenced bacterial cell growth and also the production of various enzymes and metabolites (Singh et al., 2016). Enzyme production peaked when the bacteria were cultured in the production medium containing 1.5% maltose (653 ± 7.6 U/mL) (Fig. 1c). At maltose concentrations of 2% and 2.5%, enzyme production was affected due to catabolic repression. In one study, (Pan et al., 2019) reported the enhanced production of fibrinolytic enzymes when the marine *Bacillus subtilis* was cultured in production medium containing maltose at a concentration of 1.5%. Among the selected nitrogen sources, yeast extract (642 ± 5.9 U/mL) and beef extract (610 ± 13 U/mL) enhanced enzyme yield relative to the control (487 ± 7.2 U/mL; Fig. 2a). Recently, Pan et al. (2019) reported yeast extract and tryptone as optimal medium compo-

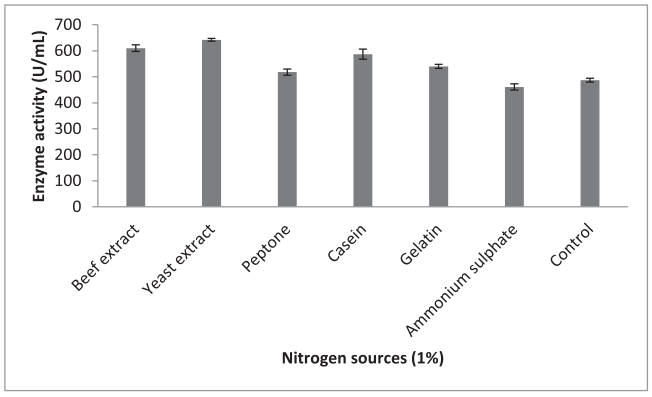

Fig. 2a. Effect of nitrogen sources on fibrinolytic enzyme production in submerged fermentation. About 1% nitrogen sources were incorporated into the production medium, which was incubated for 24 h. Error bar represents standard deviation.

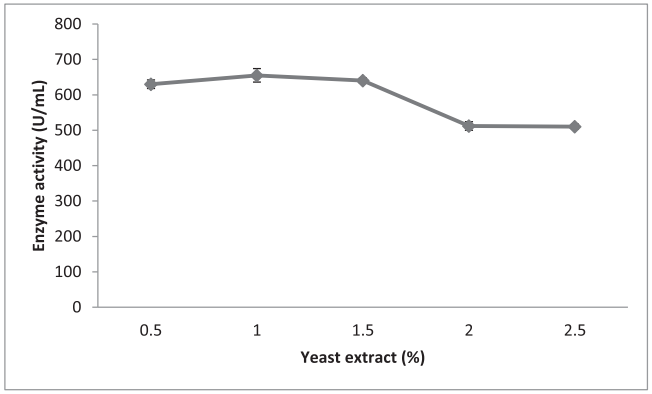

Fig. 2b. The effect of yeast extract concentrations on fibrinolysis enzyme production in submerged fermentation. Yeast extract was incorporated into the production medium, which was incubated for 24 h. Error bar represents standard deviation.

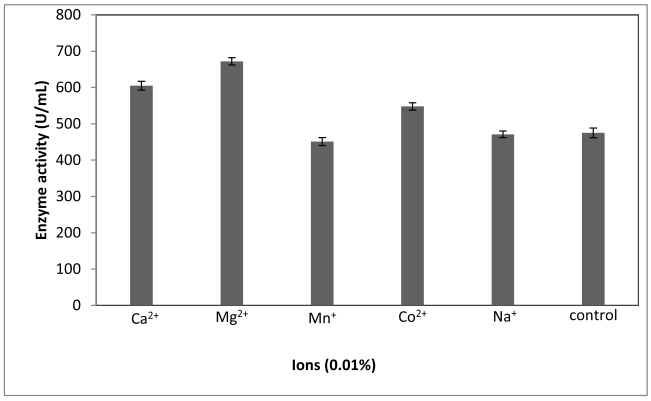

Fig. 3a. Effect of ionic sources on fibrinolytic enzyme production in submerged fermentation. About 0.01% ions were incorporated into the production medium, which was incubated for 24 h. Error bar represents standard deviation.

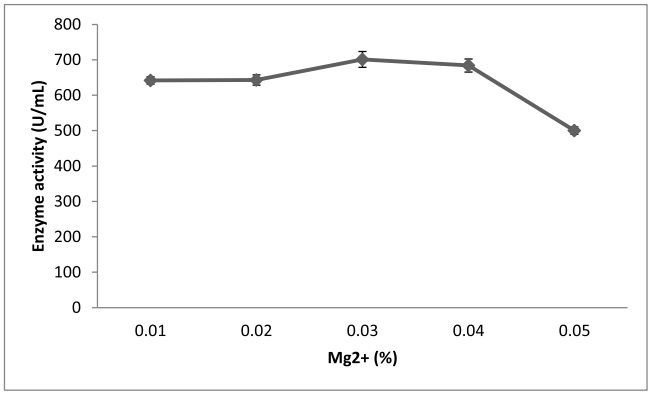

Fig. 3b. Effect of various concentrations of $MgCl_2$ on fibrinolytic enzyme production in submerged fermentation. $MgCl_2$ was incorporated into the production medium, which was incubated for 24 h. Error bar represents standard deviation.

Table 2
An analysis of variance for fibrinolytic enzyme production.

Source	Sum of	df	Mean	F-value	p-value
Model	9,924,508	9	1,102,723	495.552	<0.0001
A-Yeast extract	30.63518	1	30.63518	0.013767	0.908919
B-Maltose	1,830,560	1	1,830,560	822.6343	6.18E-11
C-$MgCl_2$	1,105,291	1	1,105,291	496.7061	7.43E-10
AB	1,306,536	1	1,306,536	587.1434	3.26E-10
AC	3,260,181	1	3,260,181	1465.091	3.53E-12
BC	424581.1	1	424581.1	190.8022	7.7E-08
$A\hat{A}^2$	1,114,896	1	1,114,896	501.0224	7.12E-10
$B\hat{A}^2$	643551.8	1	643551.8	289.2053	1.04E-08
$C\hat{A}^2$	204338.3	1	204338.3	91.82745	2.35E-06
Residual	22252.42	10	2225.242		
Lack of Fit	448.9182	5	89.78365	0.020589	0.999693
Pure Error	21803.5	5	4360.7		
Cor Total	9,946,761	19			

nents for fibrinolytic enzyme production. Yeast extract was supplemented at various concentrations and 1% was found to be optimal for enzyme production (655 ± 19 U/mL; Fig. 2b). Casein also stimulated the production of fibrinolytic enzymes (587 ± 19 U/mL). In *Pseudomonas aeruginosa* KU1, 0.1% skimmed milk enhanced the production of fibrinolytic enzymes (Kumar et al., 2018). Fibrinolytic enzymes are inducible enzymes, so the incorporation of protease-rich substances induced enzyme production. Similar results have been reported previously by (Quadar et al., (2009)).

The ions such as Mg^{2+} (672 ± 10.3 U/mL) and Ca^{2+} (605 ± 12.3 U/mL) showed enhanced fibrinolytic enzyme production relative to the control (405 ± 13.2 U/mL) (Fig. 3a). Among the various concentrations of Mg^{2+} used, 0.03% Mg^{2+} enhanced fibrinolytic enzyme production, but this was depleted at higher concentrations (Fig. 3b). These ions also maintain enzyme stability. The requirement for ionic substances varies widely. In a study, (Jhample et al., 2015) reported enhanced production of fibrinolytic enzymes in *Proteus penneri* SP-20 supplemented with culture medium containing $MnCl_2{\cdot}4H_2O$ The perfect concentration of MgSO4 for the production of fibrinolytic enzymes by Bacillus sp. according to recent study to be 0.093%. IND12 (Vijayaraghavan et al., 2017).

3.3. Optimization of fibrinolytic enzyme production by statistical method

To optimize medium components, CCD and response surface methodology (RSM) are commonly utilized. This method has several advantages over conventional optimization methods (Chen et al., 2007; Liu et al., 2005). Recently, fibrinolytic enzymes have been optimized using various statistical approaches. In addition to the nutrient sources, minerals, temperature, and pH also critically affect the production of fibrinolytic enzymes (Mahajan et al., 2012). Optimization of enzyme production through the conventional method is strenuous and tedious (Deepak et al., 2010). RSM is a classical statistical tool used for analyzing more than one factor simultaneously and the interaction between selected variables (Box and Draper 1959). In the present investigation, optimized medium showed an enhanced enzyme yield compared to previous reports from *Bacillus subtilis* A26 (Agrebi et al., 2009) and *Bacillus* sp. strain AS-S20-I (Mukherjee and Rai, 2011). Two-level full factorial experimental designs have been widely used to screen the important variables and CCD has been widely used to identify the optimal concentrations of the selected variables. Initial screening revealed that components such as yeast extract, maltose, and $MgSO_4$ were significant ($p < 0.05$). Further optimization of medium components was performed using CCD. The fibrinolytic enzyme yield varied widely and enzyme production peaked in run 7 (Table 1). The predicted R^2 value was 0.9965, which strongly agreed with the adjusted R^2 (0.9957); the difference was less than 0.2, indicating the accuracy of the designed model. Given its adequate precision, the model has been widely used to measure the signal-to-noise ratio. The obtained signal-to-noise ratio was more than 4 (76.107), indicating an adequate signal. The F-value of the designed model (CCD) was 495.55, which clearly implies that the designed model was significant (Table 2). The p value of all terms was significant ($p < 0.05$), indicating that all factors interacted and stimulated enzyme production. The lack of fit F-value was more than 0.05, indicating the robustness of the designed model.

3D response surface plots are produced as graphical illustrations of the interactions between two selected variables and also used to detect the optimal concentration for the enhanced production of enzymes. 3D graphs showed the interaction among the selected three variables (Fig. 4a–c). A 3D RSM graph provides a very simple way to understand interactions between variables, as well as the optimal levels between them for optimum enzyme production. (Surwase et al., 2012). Elliptical or circular order RSM

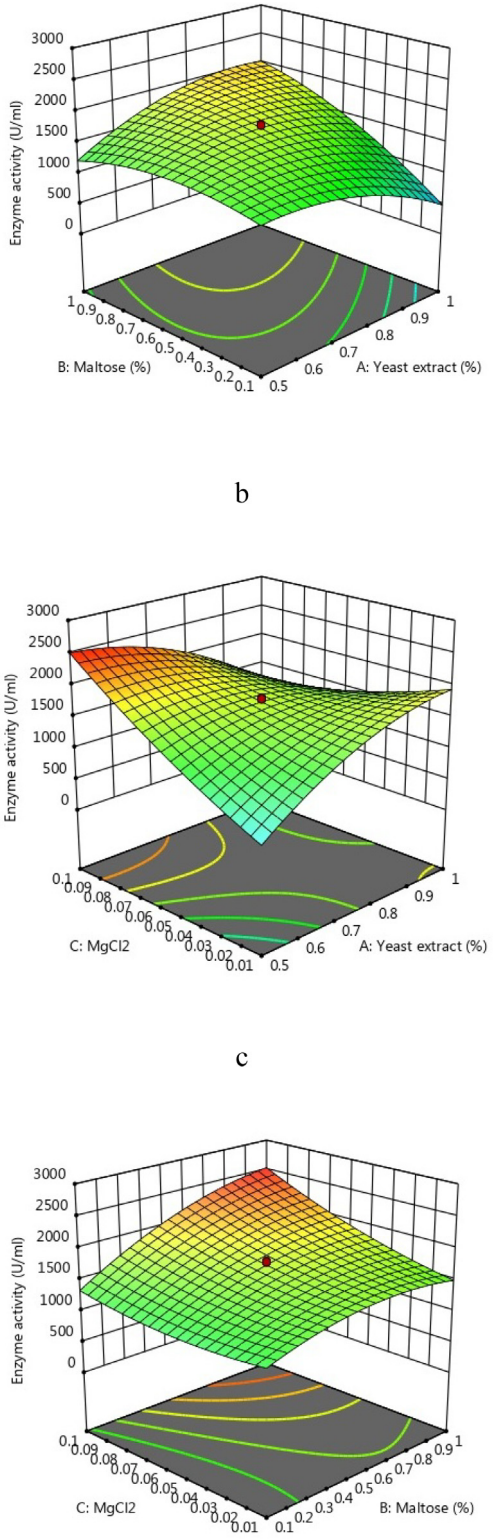

Fig. 4. 3D surface revealed the interactions between maltose and yeast extract (a), yeast extract and $MgCl_2$ (b), and maltose and $MgCl_2$ (c).

plots imply interactions between variables. In this study, the interactions between yeast extract and $MgCl_2$ were found to be more significant than those of the other tested variables. In this study, enzyme yield increased over three-fold than unoptimization. Threefold relative to that without optimization. As a result of this study, the yield of fibrinolytic enzymes was higher than that of

Bacillus sp. enzymes. *Bacillus subtilis* AS-S20-I (Agrebi et al., 2009) and AS-S20-I (Mukherjee and Rai, 2011). The selected factors remarkably enhanced the production of fibrinolytic enzymes in submerged fermentation. Our results show that optimizing the medium composition using two-level full factorial design and CCD of RSM increased the enzyme yield. The enzyme yield increased from 451 to 2559 U/mL using the optimized medium.

3.4. Human blood clot lytic activity of fibrinolytic enzyme

The crude fibrinolytic enzyme completely hydrolyzed the fibrin blood clot within 7 h at 32 ± 1 °C. *Bacillus subtilis* ICTF-1 has been demonstrated to break up blood clots in vitro with fibrinolytic enzyme (Mahajan et al., 2012). In addition, according to (Yuan et al., 2012). In vitro and *in vivo* a fibrinolytic enzyme from *Bacillus subtilis* LD-8547 hydrolyzed blood clots. Recent studies have also purified and characterized the fibrinolytic enzyme from Bacillus sp. IND7. The purified enzyme completely digested a blood clot in vitro (Vijayaraghavan et al., 2016). It is widely accepted that in vitro blood clot lysis is a reliable method of evaluating fibrinolytic agents' thrombolytic activities (Prasad et al., 2006).

4. Conclusion

An enzyme-producing bacterium that produces fibrinolytic enzymes was characterized in the present study, and it was identified as *Brevibacterium sp*. In the early stages, the variables were screened through one variable at a time; then, the central composite design was used to improve yield and determine maximum enzyme production. The fibrinolytic enzyme obtained from *Brevibacterium sp*. has great potential to lyse blood clots in vitro. The fibrinolytic enzymes from bacteria could be effectively used as alternative thrombolytic agents because commercially available thrombolytic agents have various side effects.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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