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Process optimization for production of a fibrinolytic enzyme from newly isolated marine bacterium *Pseudomonas aeruginosa* KU1

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ABSTRACT

A potent fibrinolytic enzyme producing bacteria, *Pseudomonas aeruginosa* KU1 was isolated from marine sediments of Ezhara beach, Kannur, Kerala. Enzyme production was optimized using statistical approach. Placket-Burman factorial design was used in picking key factors (Tryptone, skimmed milk and inoculum size) that influence fibrinolytic enzyme production of the isolate and further optimized using Box-Benhken design. Optimal concentrations for the selected independent variables in the medium were identified to be 0.72% w/v tryptone, 0.09% w/v skimmed milk and 3.95% v/v inoculum size. Peak production was achieved empirically in shake flask culture and it was very close to the projected activity by the response surface model. The Peak production showed 3.25 fold increase over the activity prior to any optimization and a maximum of 1.32 fold increase of one factor at a time optimization. Though many reports are available on fibrinolytic enzyme production from *Pseudomonas* sp. media optimization studies for enhancement of fibrinolytic enzyme production has not been performed so far. This may be the first report on statistical optimization of production of a fibrinolytic enzyme from marine *Pseudomonas* sp.

1. Introduction

Thrombosis is one of the major cardiovascular diseases caused by accumulation of fibrin clot inside the blood vessels and its treatment involves removal of clot. A few of the therapeutic strategies for cardiovascular diseases include usage of anticoagulants or antiplatelets. But, they may not directly act on existing clot. Other approaches are, surgical treatment or fibrinolytic enzyme therapy by which thrombus could be removed. Hence, thrombolytic enzymes are also known as clot buster enzymes. Some of these enzymes comprise tissue-type plasminogen activator (t-PA) (Collen and Lijnen, 2004), urokinase (Duffy, 2002), nattokinase (Sumi et al., 1987), lumbrokinase (Mihara et al., 1991) etc. Some of such enzymes are FDA approved for treatment of cardiovascular diseases. However, they may cause side effects such as haemorrhage, nausea etc. (Kumar and Sabu, 2017). Though fibrinolytic enzymes are reported from various sources, including microbial proteolytic enzymes (Sumi et al., 1995; Agrebi et al., 2009; Deepak et al., 2010) and in the pipeline towards its clinical application, exploration of natural resources for potential microbes with fibrinolytic potential is an impulse to bio-pharmaceutical sector.

Cost of enzyme production is one of the challenging factors regarding industrial sector. Despite availability of many fibrinolytic proteases, the expense of these enzymes with respect to production is the limitation for its widespread use. Optimization of bioprocess parameters is highly significant in reducing the overall production cost of the enzyme. Traditional one-factor-at-a-time method of optimization is laborious, time consuming. Moreover, interactions among the variables cannot be accounted while applying this method. Alternate approach for the process optimization is statistical methods which can study multiple factors simultaneously for optimization of process. When the response or output is influenced by multiple variables, the optimization can be achieved through a collection of statistical techniques referred to as response surface methodology (RSM). Appropriately planned set of experiments with statistical optimization not only reduces the time and cost but also finds optimum values of the factors under consideration (He et al., 2004; Montogomery and Myers, 2002).

Process optimization for fibrinolytic enzyme production using statistical methods was performed in case of a few bacterial species and most of them belong to *Bacillus* sp. (Wang et al., 2006; Deepak et al., 2008; Mahajan et al., 2012; Vijayaraghavan and Vincent, 2014c; Liu et al., 2005; Mukherjee and Rai, 2011). Statistical techniques applied for optimizing fibrinolytic enzyme production from bacteria, other than *Bacillus* sp. are *Psuedoalteromonas* IND11 (Vijayaraghavan and Vincent, 2014b), *Proteus penneri* (Jhample et al., 2015), *Streptomyces* sp. (Silva et al., 2015), *Paenibacillus* sp. (Vijayaraghavan and Vincent, 2014a), *Shewanella* sp. (Vijayaraghavan and Vincent, 2015), *Serratia* sp. (Taneja

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et al., 2017), etc.

Marine environment is a treasure for bioactive molecules especially enzymes. It is believed they could have lower or no toxicity or side effects due to their living in environment similar to human blood plasma chemically (Mahajan et al., 2012; Sabu, 2003). Some attempts on isolation of organisms with fibrinolytic activity from marine habitat include *Bacillus* sp. (Mahajan et al., 2012), *Alteromonas piscicida* (Demina et al., 1990) and *Pseudoalteromonas sp*. IND11 (Vijayaraghavan and Vincent, 2014b).

One of the main objectives of the present study was isolation of marine bacterium with fibrinolytic potential from the coastal region of Kannur, Kerala, since the area was hardly explored for isolation of thrombolytic enzymes. By exploring the coastal area of Kannur, new bacterial strains with significantly high fibrinolytic activity were expected. Also, the process optimization using RSM for large scale production of the therapeutic enzyme from the newly isolated marine bacterium is also focused.

2. Materials and methods

2.1. Isolation and Identification of fibrinolytic enzyme producing bacteria

Proteolytic bacteria were isolated from marine sediments of Ezhara beach (coordinates 11°49'09.9"N 75°25'02.9"E), Kannur District, Kerala, by spread plate method on skimmed milk agar. The isolated proteolytic strains were screened for fibrinolytic activity on fibrin agar plates (Astrup and Mullertz, 1952). Fibrinogen 5 mg/mL was mixed with 100 NIH units thrombin along with agar to form fibrin agar plate. 24 h old culture was centrifuged at 7000 ×*g* for 15 min and crude supernatant was spotted on fibrin agar plate. Standard fibrinolytic enzyme subtilisin was used as positive control. Those showing clear zone on fibrin agar plate were selected. Morphological and biochemical characteristics of the strain were checked and compared with the Bergey's Manual of Determinative Bacteriology, for identification of the isolate (Holt et al., 1994). The results were confirmed by biochemical and molecular methods of identification. Total genomic DNA of selected strain was isolated using genomic DNA isolation kit (Sigma, USA). PCR amplification of 16 S ribosomal RNA gene was carried out by forward primer 8 F: 5′-AGAGTTTGATCMTGG-3′ and 1492 R: 5′-ACCTTGTTACGA CTT-3′ reverse primer. PCR conditions used were, initial denaturation at 94 °C for 5 min followed by 29 cycles of denaturation at 94 °C for 30 s, annealing for 55 °C for 30 s and extension at 72 °C for 45 s and final extension at 72 °C for 10 mins. Nucleotide sequencing of amplified product was carried out at Eurofins (Bangalore). Sequence similarity search was performed using NCBI BLAST against non-redundant nucleotide (nr/nt) database in the NCBI GenBank (www.ncbi.nlm.nih.gov).

2.2. Production of fibrinolytic enzyme

Production of fibrinolytic enzyme was carried out in minimal salt medium containing KH_2PO_4, $(NH_4)_2SO_4$, $CaCl_2{\cdot}2H_2O$, $MgSO_47H_2O$ and NaCl (0.005% w/v) supplemented with 1% w/v glucose and yeast extract. pH of the medium was adjusted to 7. Seed culture was prepared in Luria Bertani (LB) broth. After 12 h of incubation 1% (v/v) inoculum was transferred to the above mentioned production medium and incubated for 48 h. Culture was centrifuged at 7000 ×*g* for 30 min to isolate crude enzyme.

2.3. Assay of enzyme activity

200 µL of 0.72% fibrinogen and 100 µL of thrombin (20 U/mL) were mixed with 700 µL of Tris-HCl buffer (20 mM pH 7.4) to form fibrin clot. 100 µL of enzyme was added to this clot and incubated for 1 h at 37 °C. 1 mL of 10% trichloroacetic acid (TCA) was added and kept for 20 min, followed by centrifugation at 3000 ×*g* for 15 min. Optical density of supernatant collected was measured at 275 nm and converted to tyrosine equivalent. One fibrinolytic unit is defined as µ moles of tyrosine released per mL per hour (Deepak et al., 2008).

2.4. Preliminary optimization using one factor at a time method

Initial parameter optimization was carried out using traditional one factor at a time method. Various Physico-chemical parameters, as well as medium components, were screened for maximal production. Factors including incubation time, incubation temperature, initial medium pH, agitation rate (rpm), inoculum size, carbon source, nitrogen source and metal ions on production of enzyme were evaluated and range of values for each factor (minimum/maximum) was determined. Effect of skimmed milk on production of the enzyme was also evaluated.

2.5. Statistical optimization

2.5.1. Screening of factors influencing production using Plackett–Burman's (P-B) factorial design

The Plackett-Burman factorial design aids to screen a large number of variables and determine active factors with fewer experimental runs (Plackett and Burman, 1946). From the results obtained, optimization using one-factor-at a time method, 8 factors were chosen for Plackett–Burman's factorial design. Selected factors with their corresponding range of values are, lactose-most suitable carbon source (0.05–0.1% w/v), tryptone-most suitable nitrogen source (0.1–1% w/v), skimmed milk (0.05–0.1% w/v), KH_2PO_4 (0.002–0.02% w/v), $(NH_4)_2SO_4$ (0.0018–0.018% w/v), agitation rate (100–250 rpm), time of incubation (24–48 h) and inoculum size (1–5% v/v). All the factors were examined with respect to its low (-) and high levels (+) using P-B design (Table 1). 12 combinations were run for the factors and each trail was carried out in triplicate. Plackett–Burman's design is based on the first-order polynomial model:

$$Y = \beta_0 + \sum \beta i Xi \quad (1)$$

where Y is the response (fibrinolytic activity of enzyme in Units/mL), β_0 is the model intercept and βi is the linear coefficient for independent variable Xi. Though P-B design analyzes the major factors influencing response, it doesn't study the interaction among factors. Regression analysis was performed and significant factors were determined based on positive linear coefficient and P value less than 0.05. Those showing negative linear coefficient were kept at mid-level for further optimization and subsequently, those with positive linear coefficient with P value greater than 0.05 were also kept at mid-level. Significant factors thus determined were further optimized by response surface methodology (RSM) using Box-Behnken design.

2.5.2. Response Surface Methodology (RSM) using Box-Behnken design

RSM approximates the true relationship among the independent variables and response using lower order polynomials (first or second). They not only consider the linear relationship between the variables but

Table 1
Variables for P-B design with coded levels.

Factors	Units	Coded Levels	
		− 1	+ 1
Lactose	%	0.05	0.1
Tryptone	%	0.1	1
Skimmed milk	%	0.05	0.1
KH_2PO_4	%	0.002	0.02
$(NH_4)_2SO_4$	%	0.0018	0.018
Agitation rate	Rpm	150	250
Time	Hours	24	48
Inoculum size	%	1	5