Regulation of Process Parameters for Improved Synthesis of Thrombolytic Enzyme from *Bacillus cereus* S46

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Received 03 July 2018; revised 17 January 2019; accepted 23 April 2019

The study aimed to isolate soil bacteria and screen them for thrombolytic activity. Effect of process parameters on enzyme production were analyzed using one factor at a time approach. Isolate S46 demonstrated highest blood clot dissolution (15.64%) and was chosen for further study. Based on the partial 16S rDNA sequence, isolate S46 was identified as *Bacillus cereus*. Addition of sucrose and casein (1% w/v) to the broth resulted in highest enzyme yield (130 U/ml). Enzyme production improved in presence of MgSO$_4$ (0.08%, w/v), FeSO$_4$ (0.004% w/v), respectively. Enzyme production was best (186.66 and 210.86 U/ml) at pH 8.0 and 30 °C. Maximum enzyme production (220.86 U/ml) was reported at 72 h with inoculum size of 1.5% v/v. The above conditions advocate that media optimization enhanced the thrombolytic enzyme yield by *Bacillus cereus* S46.

Keywords: *Bacillus cereus*, thrombolytic enzyme, submerged fermentation, process optimization

Introduction

Thrombosis is a clinical condition where undesirable blood clots occur inside the blood vessels and is one of the leading causes of death worldwide$^1$. Some of the recent thrombolytic drugs used are streptokinase, urokinase, tissue plasminogen activator and single-chain urokinase-type plasminogen activator$^2$. The economic feasibility, high yields, consistency, ease of product modification and optimization, greater catalytic activity, etc. have favoured the use microbial thrombolytic enzymes to be formulated as drugs for prevention and cure of cardiovascular complications$^3$. Among the various microbial sources, the genus *Bacillus* has gained much importance due to its lack of endotoxins, short fermentation cycles, and ability to produce several proteases with some species being considered safe in drug administration$^4$. The present study aims to isolate *Bacillus* species from soil and evaluate the various process parameters supporting maximal thrombolytic enzyme production.

Materials and methods

Sample collection and isolation of potential thrombolytic enzyme producing bacteria

Soil samples collected from various regions of Bangalore and Andhra Pradesh, India. were segregated and dried overnight in a hot air oven (set at 55 °C) for complete moisture removal. The samples were serially diluted and suspensions plated on nutrient agar followed by incubation at 37 ± 2 °C for 24 h.

Screening of thrombolytic enzyme producing bacteria

Isolates were cultured on casein agar (1% w/v casein, pH 6.8 ± 0.2) and observed for zone of caseinolysis. Enzymatic index was calculated by dividing width of zone of caseinolysis by width of the colony. Isolates showing enzymatic index ≥ 1.5 were selected for further screening. Secondary screening involved the dissolution of artificial blood clot *in vitro* according to the protocol of de Souza *et al.$^5$. The percentage of clot lysis was calculated according to the equation,

\[
\text{Percentage lysis} = 100 - \left\{ \frac{(W3 - W1)}{(W2 - W1)} \right\} \times 100
\]

where, W1 is the weight of the empty tube, W2 is the weight of tube with clot, W3 is the weight of tube after clot lysis.

Identification of thrombolytic enzyme producing bacteria

DNA was amplified by PCR containing the template DNA (1 μl), forward and reverse 16S rDNA primers (400 ng: 5’ GTAGTCATATGCTTGTCTC-3’ and 5’ GAAACCTTGTTACGACTT-3’, respectively), dNTPs (4 μl, 2.5 mM each), Taq DNA polymerase assay buffer (10 μl), Taq DNA polymerase (1 μl, 3 U/μl). The Thermal Cycler (ABI 2720, Applied
The isolate yielding the highest enzyme activity was grown in mineral salt medium of Bhardwaj et al.\(^7\) (g/l: KH\(_2\)PO\(_4\) 2.5; MgSO\(_4\).7H\(_2\)O 0.4; NaHCO\(_3\), 1.0; C\(_2\)H\(_5\)NaO\(_5\), 1.0; FeSO\(_4\), 0.02; MnCl\(_2\), 0.02; pH 7.5 ± 0.2) supplemented with different carbon sources such as 1% (w/v : lactose, fructose, glucose, mannitol, sucrose, maltose or soluble starch), nitrogen sources 1% (w/v : casein, yeast extract, tryptone, peptone, beef extract, ammonium acetate, ammonium chloride ammonium sulphate, sodium nitrate or ammonium ferrous sulphate), MgSO\(_4\) (0.02 to 0.1% w/v, with the increment of 0.02%), FeSO\(_4\) (0.002 to 0.01% w/v, with the increment of 0.002%). The various physical parameters considered were initial pH (4.0, 5.0, 6.0, 7.0, 8.0 or 9.0), temperature (25, 30, 35 or 40 ± 2 °C) incubation period (24, 48, 72, 96 or 120 h), inoculum load (0.5 to 3.0% v/v, with an increment of 0.5%) and agitated and static culture conditions using one factor at a time approach for maximal enzyme production.

**Optimization of physico-chemical parameters**

The isolate yielding the highest enzyme activity was analyzed using Seq Scape (version 5.2). The bacterial isolate was identified by comparing the sequence with known 16S ribosomal sequences in the NCBI database using BLASTn. The nucleotide sequence was submitted to GenBank database (NCBI, USA) and was provided an accession number\(^6\).

**Enzymatic assay**

Protocol of Gessesse et al.\(^8\) with minor modifications was adopted. The cell free supernatant (100 µl) was considered as crude enzyme extract and was coupled with casein (1% w/v, 500 µl, prepared in phosphate buffer, pH 7.5). The reaction mixture was incubated at 37 °C for 15 min. Trichloroacetic acid (5% w/v, 2.5 ml) was used to arrest the reaction by incubating at 37 °C for 10 min. It was then centrifuged (10,000 rpm, 4 °C for 5 min), supernatant was treated with a solution of sodium carbonate (6% w/v, 2 ml) and dilute Folin- Ciocalteu reagent (500 µl) in a ratio of 1:2 (FC reagent: water). Absorbance was measured at 660 nm using a spectrophotometer (Shimadzu UV spectrophotometer UV-1800). One unit enzyme activity (U/ml) was defined as the amount of enzyme releasing 1 µ mol of tyrosine equivalent/min under standard assay conditions.

**Statistical analysis**

Effect of each parameter affecting enzyme production was studied in duplicate and the data was graphically presented as mean ± S.D. (displayed as Y-error bars in the figures).

**Results and Discussion**

Isolation and screening of thrombolytic enzyme producing bacteria

A total of 62 bacterial isolates were obtained of which 28 isolates showed zone of caseinolysis. Eight isolates were compliant with an enzymatic index ≥1.5 and were further evaluated for blood clot dissolution assay. Isolate S46 showed an enzymatic index of 3.42 with 15.64 % of clot dissolution and was selected for further analysis.

**Identification of thrombolytic enzyme producing bacteria**

A 706 bp 16S rDNA nucleotide sequence was obtained by PCR and related with 16S ribosomal sequences available on NCBI database using BLASTn. The isolate S46 exhibited high sequence similarity (99%) to that of Bacillus cereus ATCC 14579 and was designated as Bacillus cereus S46. The gene sequence was provided a Gene Bank accession number MH392725.

**Effect of different carbon supplements**

Carbon source besides acting as a major cell membrane constituent, also act as an energy source for various activities. They are able to affect the anaerobic pathway of the microorganism and subsequently their enzyme activity\(^9\). Sucrose gave the highest enzyme yield (125.8 U/ml) among the carbon sources tested (Figure.1a). This could be due to the hydrolysis of sucrose to fructose and glucose making availability of more than one carbon source possible. Literatures suggest that when bacteria have the availability of more than one sugar, they prefer to use an easily metabolizable sugar to enter the first exponential growth phase. Subsequently, they enter a short lag phase of non-growth, a period called the diauxic lag phase where the utilization of the secondary sugar molecules occur. This leads to the initiation of the second exponential phase\(^10\). Supplementation of sucrose in the medium yielded maximal production of protease (155.38 U/ml) in B. megaterium IBRL MS 8.2\(^11\).
Effect of nitrogen supplementation

Microbial metabolism of nitrogen produces amino acids, nucleic acids, proteins, components of the cell wall and regulates enzyme synthesis. They also decrease acetic acid’s inhibitory effect (generated from sugar metabolism further affecting the pH) and in turn influence enzyme activity and stability.

Casein supplementation resulted in highest enzyme production (130 U/ml) by B. cereus S46 (Figure 1b). Casein is considered as a highly nutritive source of energy, where the peptide bond cleavage leads to the liberation of essential amino acids (leucine, isoleucine and valine) to be used in protein synthesis. Maximum protease yield (1057 U/ml) was reported from B. amyloliquefaciens An6 using casein.

Optimization of percentage of MgSO4

Besides being a cofactor for intracellular reactions, inorganic compounds contribute to metabolite structures of the enzymes. Consequently, combined with their anions, the essential cations strongly affect formation of metabolites by changing metabolic fluxes and influencing biological pathways. MgSO4 at a concentration of 0.08% (w/v) boosted enzyme production (164.33 U/ml) while a concentration of 0.1% w/v resulted in a decreased in enzyme activity. Magnesium ions reportedly play critical roles in cellular metabolism by adhering to the protein surface, nucleic acids and cell membranes thereby stabilizing them. They are also integral to the enzyme’s structural and catalytic activities. Parallel to the results of the present study, supplementation of culture medium with MgSO4 (0.2%) was found to improve thrombolytic enzyme production in B. cereus NK-1.

Optimization of percentage of FeSO4

A gradual increase in enzyme yield was observed from 0.002% (w/v) FeSO4, with maximum yield (172 U/ml) obtained at 0.004% (w/v). Oxidative stress, induced by the presence of ferrous iron (in excess) in the broth during the production of thrombolytic enzyme by B. cereus S46, may have led to a decline in enzyme production. Otherwise, complex formation of ferrous iron with thiols such as cysteine can generate free radicals (hydroxyl) via Fenton chemistry, which could in turn initiate lipid peroxidation. This primarily alters the membrane properties and decreases membrane fluidity, thus disrupting membrane-bound proteins significantly.

Effect of initial pH of the medium

A steady increase in enzyme secretion was observed till the optimum was reached at pH 8.0 yielding 186.66 U/ml. Drop in enzyme activity was recorded across extreme acidic (pH 4.0, 103.2 U/ml) and alkaline pH (pH 9.0, 165 U/ml) indicating that both these conditions were inhibitory to growth and enzyme secretion (Figure 2a). Since, the cell membrane gets involved in the transport of nutrients; any instability in terms of the ionic potential also disrupts the nutrient channeling. At extremes of pH, bacterial cells generate proton motive force to counteract the ionic potential imbalance across the cell surface. Hence, to maintain the internal pH of the organism, a part of the reserved cellular energy (which would otherwise be utilized for vital metabolic processes) is spent. The optimum culture pH for B. subtilis KCK-7 was also reported as pH 8.0.

Effect of agitation

A comparison between the effect of an agitated culture and a static culture suggests that an agitated
culture produced more enzyme (190.86 U/ml) as opposed to when the culture remained static (103.6 U/ml). Literature suggests that agitation of culture medium increases the generation of sugars which consequently helps the microbial synthesis of enzymes. Besides, agitation also increases the dissolved oxygen supply in the cultivation medium. Nutrient uptake by bacterial cells also increases protease production.

In *B. licheniformis* the highest protease production and growth were obtained when agitation while the static condition almost inhibited its production. In a similar study, maximum protease production (88.0 U/ml) in *B. megaterium* IBRL MS 8.2 was achieved at 150 rpm.

**Effect of load of inoculum**

The maximum enzyme produced (201.66 U/ml) corresponded to an inoculum load of 1.5% v/v. Inoculum size when increased beyond the optimum level, decreased enzyme production (Figure 2b). While using lesser inoculum, the higher surface area of bacteria to volume ratio is considered to be responsible for enhanced thrombolytic enzyme production. Additionally, effective nutrient uptake and improved dissolved oxygen distribution might have contributed to a higher enzyme yield. Larger inoculum sizes might have led to depletion of nutrients and oxygen in the broth. The greatest thrombolytic protease activity (22.10 ± 0.80 U/ml) by *Bacillus cereus* GD55 was registered at 2.0% of inoculum level. Yet, at 5.0% of the inoculum level minimum amount of thrombolytic protease (10.42 ± 0.20 U/ml) was observed.

**Effect of incubation temperature**

The effect of temperature on thrombolytic enzyme production by *B. cereus* S46 suggest that an incubation temperature of 30 °C was most suitable as maximum yield (210.86 U/ml) was obtained at this temperature. Temperature greater than the optimum decreased the enzyme production where, at 35 °C and 45 °C it was recorded as 198.45 U/ml and 140 U/ml, respectively (Figure 3a). Maximum protease production in *B. pumilus* ATCC7061 was noted at 30 °C. At higher temperatures, enzyme production decreased because the enzyme suffered from thermal inactivation.

Fig. 2 — Effect of physical parameters on fibrinolytic enzyme production by *B. cereus* S46 (a) initial pH of the medium, (b) load of inoculum. Data represent mean ± S.D. (n=2)

Fig. 3 — Effect of physical parameters on fibrinolytic enzyme production by *B. cereus* S46 (a) incubation temperature, (b) incubation time. Data represent mean ± S.D. (n=2)
Effect of incubation time

Incubating the *B. cereus* S46 culture for 72 h resulted in highest enzyme synthesis (220.86 U/ml) and a subsequent decrease in enzyme generation was noted with 96 h (190.66 U/ml) and 120 h (170.46 U/ml) of incubation time (Figure 3b). The decrease in enzyme yield could possibly be due to faster depletion of the functional components of the media and developed oxygen tension. The accumulation of excess acid from sugar utilization also referred to as the Crabtree effect, can also contribute to a drop in enzyme yield. Excess acid accumulation in the medium generates toxic byproducts and inhibits the cellular growth, thereby limiting protein yield. Parallel results were obtained for thrombolytic protease from *Bacillus sp. IND12*.

Conclusions

The present study suggests that *B. cereus* S46 is a potential candidate for the thrombolytic enzyme production and cultural parameters have a profound effect on the enzyme synthesis. However, in order establish its footprints in the pharmaceutical industry, the thrombolytic enzyme should undergo several purification regimes and be well characterized for better understanding of its properties.

Acknowledgements

We extend our sincere gratitude to the management of Jain (Deemed-to-be University) for providing the research facilities."

References

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