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Optimization of Fibrinolytic Enzyme Production from *Bacillus* sp. IND6 in Solid State Fermentation

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Authors' contributions

This work was carried out in collaboration between all authors. Author MAA analyze the data, author DSDD performed the experiment, and author PV wrote the first draft of the manuscript. All authors read and approved the final manuscript.

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ABSTRACT

Aims: This study aimed to screen and optimize *Bacillus* sp. IND6 for fibrinolytic enzymes production. The process parameters were optimized to enhance the production of fibrinolytic enzyme in solid state fermentation using wheat bran substrate.
Methodology: In this study fibrinolytic enzyme production was carried out using *Bacillus* sp. IND6 in solid-state fermentation. Wheat bran was used as the substrate. The process parameters were optimized using traditional one-variable-at-a-time approach.
Results: The significant increase in fibrinolytic enzyme production was occurred when this organism was grown up to 72 h (1310 ± 31.6 U/g), at pH 9.0 (1417.4 ± 73.2 U/g) with 90% moisture content (1441.2 ± 77.4 U/g). Among the carbon sources, starch significantly enhanced enzyme production (1713.8 ± 53.9 U/g).
Conclusion: *Bacillus* sp. IND6 could be the good source of fibrinolytic enzymes for various biotechnological applications.

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1. INTRODUCTION

Fibrinolytic enzymes are generally considered as the novel thrombolytic substances to prevent and treat cardiovascular diseases (CVDs) [1]. The thrombolytic agents such as, tissue plasminogen activator and urokinase plasminogen activator are very expensive and are safe. Moreover, the bacterial streptokinase is considered as a cheap thrombolytic agent and causes undesirable side effects [2]. Hence, searching for a novel thrombolytic agent to treat and prevent CVDs continues. The fibrinolytic enzyme producing organisms, especially bacteria were isolated from many Asian foods which attracted attention worldwide [1]. The fibrinolytic enzyme production was optimized and was characterized from various sources including *Bacillus thuringiensis* IMV B-7324 [3], *Bacillus thuringiensis* IMB B-7324 [4], *Virgibacillus* sp. SK37 [5], *Streptomyces* sp. MCMB [6], *Bacillus subtilis* RJAS19 [7], and *Bacillus* sp. [8].

Solid-state fermentation (SSF) is a useful technique for the utilization of cheap agro-residues for the production of various enzymes. The agroresidues such as green gram husk [9], pigeon pea [10], sesame oil cake [11] and potato peel [12] were used as the solid substrate for the production of various commercially available enzymes. In SSF process, the solid substrates provide nutrients to the microorganisms. So, the selected solid culture medium for any enzyme bioprocess should contain essential nutrients for the production of enzymes. SSF is also useful for the production of enzymes from bacteria [13-16]. Considering the nutrient compositions, availability, wheat bran was used as the potential substrate for fibrinolytic enzyme production from *Bacillus* sp. IND6 and the process parameters were optimized.

2. MATERIALS AND METHODS

2.1 Screening of Bacterial Isolates from Fermented Rice for the Production of Fibrinolytic Enzyme

Rice was purchased from the local market and it was boiled for 50 min and incubated at room temperature for 48 h for microbial fermentation. The fermented rice was used as the source fibrinolytic enzyme producing bacterial isolates. Approximately 2.0 g of sample (fermented rice) was mixed in 100 ml of distilled water and it was further serially diluted (10^{-1} to 10^{-7}) using double-distilled water and plated on skimmed milk agar plates. These skimmed milk agar plates were incubated for 24–72 h at 37°C. After 48 h, the protease-producing bacterial isolates show a clear zone on these plates. The selected three protease-producing bacterial strains were cultured in nutrient broth medium ((g/l), casein 10, yeast extract 5, peptone 5, and NaCl 1.5). Enzyme production was carried out in 250-ml Erlenmeyer flasks, and these flasks were kept on an orbital shaker (175 rpm, 48 h, and 37°C). After 48 h of fermentation of the culture medium, all three cultures were centrifuged (10,000 rpm, 10 min) and the cell free extract was used as the crude enzyme. The fibrin plate was prepared with 50 µl thrombin (100 NIH U/ml), 1% (w/v) agarose (Himedia, Mumbai, India) and 0.5% (w/v) fibrinogen (ID Bio, France). These substances were mixed and allowed to stand for 1 h at room temperature (30°C ± 2°C) to form a fibrin clot. About 20 µl of crude enzyme from the selected bacterial isolates was dropped into the wells and incubated for 5 h (30°C ± 2°C), and the zone of hydrolysis was observed [17].

2.2 Biochemical and Molecular Identification of the Selected Bacterial Isolate

The bacterial isolate with highest fibrinolytic enzyme activity was identified based on its biochemical tests such as, motility test, catalase test, casein hydrolysis, starch hydrolysis, urea hydrolysis, nitrate reduction, indole test and citrate utilization [18] and 16S rDNA sequencing [19]. The 910 bp sequences were submitted to the GenBank database and an accession number was assigned.

2.3 Enzyme Assay

Fibrinolytic enzyme-producing ability of *Bacillus* sp. was assayed using fibrin substrate. Briefly, the crude sample was used for fibrinolytic enzyme determination. About 0.2 ml aliquot of crude enzyme was mixed with Tris-HCl buffer (2.5 ml, 0.1 M, pH 7.8) containing 0.01 M calcium chloride. Then fibrin (2.5 ml, 1.2%, w/v) was added and incubated (30 min, 37°C). The reaction was stopped by adding trichloroacetic acid (5.0 ml; 0.11 M) containing acetic acid (0.33 M) and sodium acetate (0.22 M). This was allowed to stand for 30 min and centrifuged

(10,000 rpm, 10 min). The absorbance was measured at 275 nm against sample blank. One unit of fibrinolytic activity was defined as the amount of enzyme that liberates 1 µg of L-tyrosine per minute under the standard assay conditions.

2.4 Fibrinolytic Enzyme Production Using SSF

Enzyme production was carried out in 100 ml Erlenmeyer flasks containing 2 g wheat bran. It was moistened with 1.5 ml buffer (Tris-HCl buffer, pH 8.0, 0.1 M). The substrate was sterilized (121°C, 20 min), inoculated with 5% inoculum and incubated at 37°C for 48–72 h. Fibrinolytic enzyme was extracted from the fermented medium by adding 20-ml double-distilled water and placed in an orbital shaker (150 rpm, 30 min, 30°C ± 2°C). It was further centrifuged at 10,000 rpm (10 min, 4°C), and the cell free extract was used as the source of crude fibrinolytic enzyme.

2.5 Optimization of Fibrinolytic Enzyme Production

The process parameters for the production of fibrinolytic enzyme were carried out by one-variable-at-a-time approach. SSF was carried out in a 100-ml Erlenmeyer flask containing 2.0 g wheat bran moistened with 1.5 ml Tris- buffer (0.1 M, pH 8.0). The process parameters such as the incubation period (12–96 h), inoculum (2%–10%), moisture content (60%–100%), pH (5.0–10.0), carbon sources (1%, w/w; maltose, sucrose, xylose, glucose, starch, and trehalose), nitrogen sources (1%, w/w; casein, yeast extract, peptone, beef extract, urea, and gelatin), and ions (0.1%, w/w, $Na_2H_2PO_4$, $MgSO_4$, $Na_2H_2PO_4$, sodium nitrate, calcium chloride, ferrous sulphate, ammonium chloride, NaH_2PO_4, and ammonium sulphate) were carried out for enzyme production. Then, about 20 ml of distilled water was mixed with the fermented medium and fibrinolytic enzyme assay was carried out.

3. RESULTS AND DISCUSSION

3.1 Fibrinolytic Enzyme Producing Bacterial Isolate

In the present study, three potent bacterial isolates were selected for fibrinolytic enzyme screening. The isolate *Bacillus* sp. IND6 showed potent fibrinolytic activity and this organism produced 11-mm zone on fibrin plate (Fig. 1). This organism was identified as *Bacillus* sp. IND6 based on biochemical test and 16S rDNA sequencing. In this study, fermented rice was used as the sample source. Fibrinolytic enzyme screening was carried out previously from various sources, including, Indonesian *tempeh* [20], Japanese *shiokara* [21], and fermented red bean [22], has been carried out.

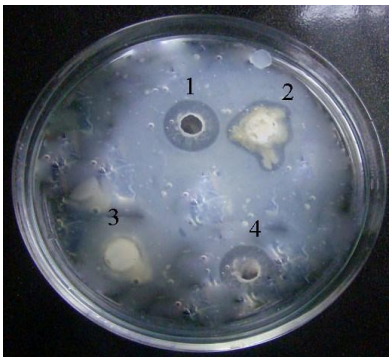

Fig. 1. Screening of fibrinolytic enzyme producing bacteria. 20 µl crude sample was loaded into fibrin plates and incubated for 5 h at room temperature. (1- crude enzyme from strain IND6; 2, 3 and 4: crude enzyme from other bacterial isolates).

3.2 Optimization of Fibrinolytic Enzyme Production

Fibrinolytic enzymes are generally produced by SSF and submerged fermentation (SmF). SSF has numerous advantages over SmF [22]. Hence, in the present study, SSF was carried out for the production of fibrinolytic enzyme. In SSF, many substrates have been used for the production of fibrinolytic enzyme. The main criteria for the selection of an ideal substrate for SSF are availability and cost [22], and nutrient composition of the substrate [23]. To evaluate the effect of incubation period on enzyme production, the selected isolated was subjected to various fermentation periods (12–96 h) and maximum fibrinolytic activity (1310 ± 31.6 U/g) was observed after 84 h incubation (Fig. 2). For further experiment, the fermentation period was maintained as 84 h. To evaluate the effect of pH for enzyme production, the fermented medium was incubated in pH ranging from 5.0 to 10.0 and maximum fibrinolytic activity was recorded at pH 9.0 (1417.4 ± 73.2 U/g) (Fig. 3). The pH of the fermentation medium was further maintained

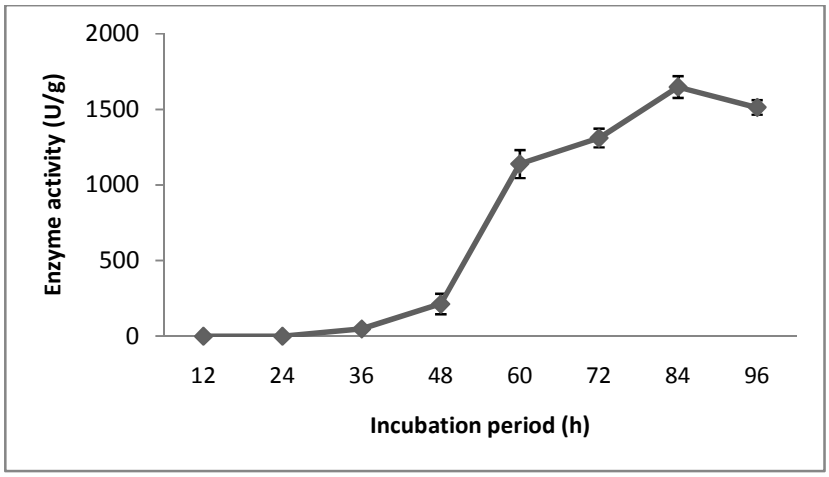

Fig. 2. Effect of incubation time on fibrinolytic enzyme production from *Bacillus* sp. IND6 in solid state fermentation

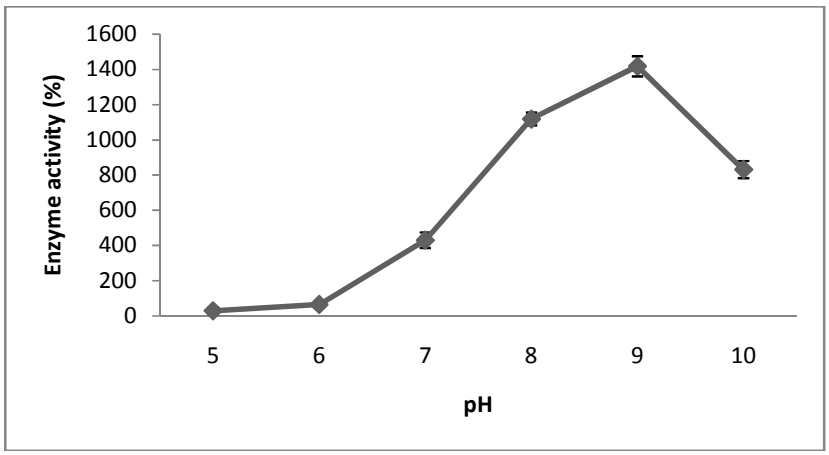

Fig. 3. Effect of pH on enzyme production from *Bacillus* sp. IND6 in solid state fermentation

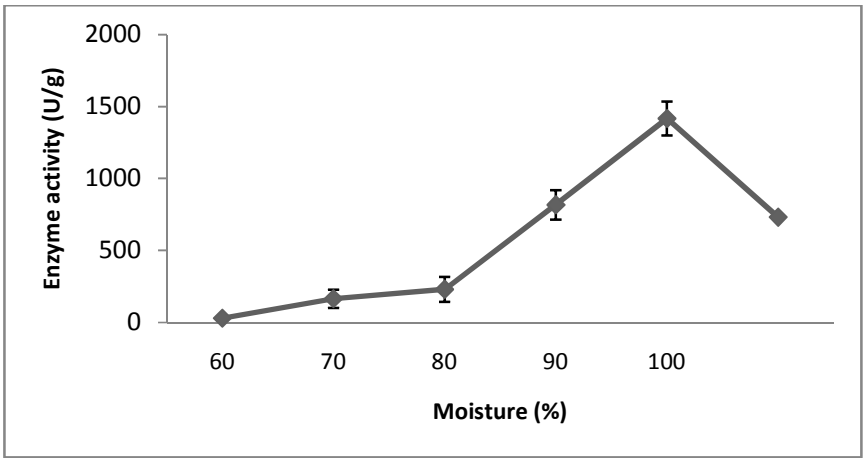

Fig. 4. Effect of moisture content on enzyme production from *Bacillus* sp. IND6 in solid state fermentation

as 9.0. In the present study, the optimum moisture content was evaluated to enhance the production of fibrinolytic enzyme and maximum enzyme activity (1441.2 ± 77.4 U/g) was observed at 90% moisture content (Fig. 4). In SSF, moisture content of the medium is one of the critical factors that significantly influence the enzyme production. The optimum moisture content for fibrinolytic enzyme production could vary depends on the substrate and organism used in SSF process [9]. In SSF microbial growth and product formation occurs at or near the surface of the solid substrate particle having low moisture contents [23].

Many carbon sources were used to determine their impacts on fibrinolytic activity of *Bacillus* sp. IND6. Maximum fibrinolytic enzyme activity was observed with the culture medium supplemented with starch (1713.8 ± 53.9 U/g). The carbon sources such as, trehalose, xylose, glucose, and maltose also enhanced enzyme production (Fig. 5). This result was in accordance with the observation made previously with other *Bacillus* sp. [24,25]. Various nitrogen sources were examined to elucidate their impacts on fibrinolytic enzyme production. The results showed that the selected *Bacillus* sp. utilized all tested nitrogen sources. Maximum enzyme production was observed with the culture medium supplemented with beef extract (1645.6 ± 38.5 U/g) (Fig. 6). Similar positive impacts of beef extract was reported in *Bacillus cereus* IND1 [26]. In most bacteria, both organic and inorganic forms of

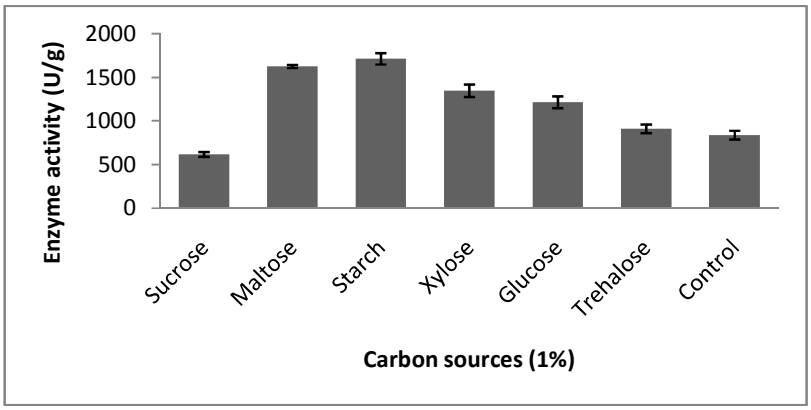

Fig. 5. Effect of carbon source on enzyme production from *Bacillus* sp. IND6 in solid state fermentation

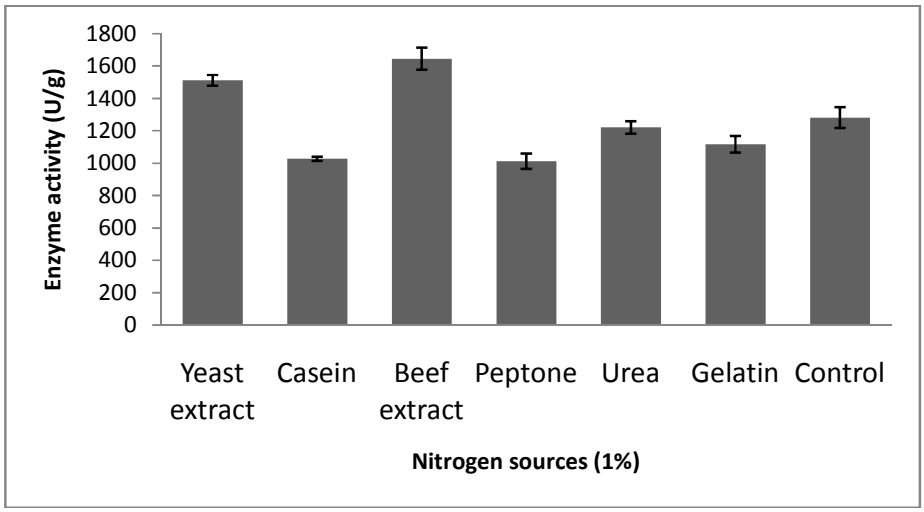

Fig. 6. Effect of nitrogen source on enzyme production from *Bacillus* sp. IND6 in solid state fermentation

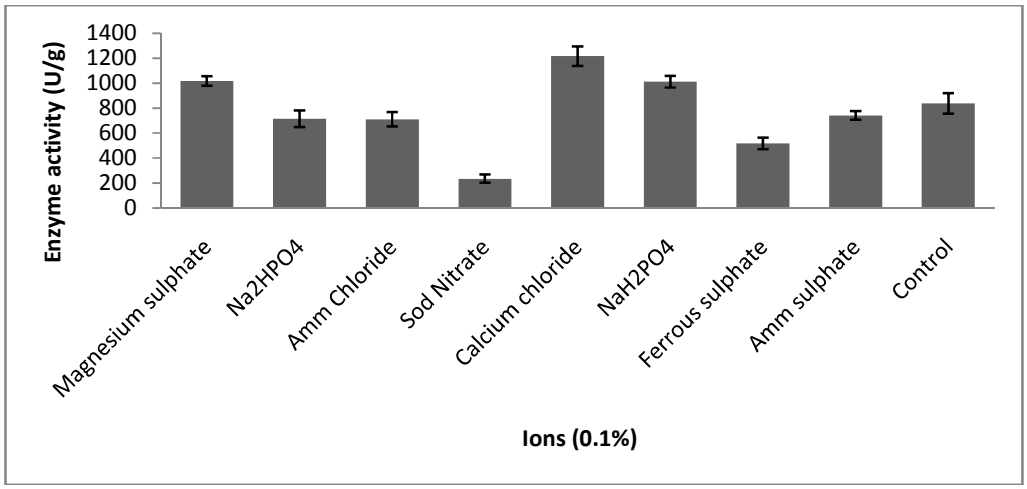

Fig. 7. Effect of ions on enzyme production from *Bacillus* sp. IND6 in solid state fermentation

nitrogen are metabolized to produce nucleic acids, amino acids, cell wall components and proteins. The alkaline protease comprises 15.6% nitrogen and its production is dependent on the availability of both nitrogen and carbon sources in the culture medium [27]. Although complex nitrogen sources are usually used for protease production, the requirement for a specific nitrogen sources differs from organism to organism.

Different mineral salt solutions were examined to determine their effects on fibrinolytic activity of the isolate (Fig. 7). Maximum fibrinolytic enzyme production was observed with calcium chloride (1217.8 ± 39.4 U/g). It was also registered previously where $MgSO_4$ has been found to be good inducer for fibrinolytic enzyme production by *Bacillus subtilis* BS-26 [28]. The optimized medium showed 1928 ± 49 U/g enzyme activity, which was about 1.5 fold higher than unoptimized medium.

4. CONCLUSION

In the present study, fibrinolytic enzyme production was optimized from *Bacillus* sp. IND6 in solid state fermentation. The process parameters were optimized and enzyme production was enhanced. The process parameters such as fermentation period, pH and moisture significantly enhanced enzyme production. These kinds of studies show the best parameter combination for fibrinolytic enzyme production.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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