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Research Article

Bioconversion of agro-industrial wastes for the production of fibrinolytic enzyme from *Bacillus halodurans* IND18: Purification and biochemical characterization

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ABSTRACT

Background: Agro-wastes were used for the production of fibrinolytic enzyme in solid-state fermentation. The process parameters were optimized to enhance the production of fibrinolytic enzyme from *Bacillus halodurans* IND18 by statistical approach. The fibrinolytic enzyme was purified, and the properties were studied.
Results: A two-level full factorial design was used to screen the significant factors. The factors such as moisture, pH, and peptone were significantly affected enzyme production and these three factors were selected for further optimization using central composite design. The optimum medium for fibrinolytic enzyme production was wheat bran medium containing 1% peptone and 80% moisture with pH 8.32. Under these optimized conditions, the production of fibrinolytic enzyme was found to be 6851 U/g. The fibrinolytic enzyme was purified by 3.6-fold with 1275 U/mg specific activity. The molecular mass of fibrinolytic enzyme was determined by sodium dodecyl sulphate polyacrylamide gel electrophoresis, and it was observed as 29 kDa. The fibrinolytic enzyme depicted an optimal pH of 9.0 and was stable at a range of pH from 8.0 to 10.0. The optimal temperature was 60°C and was stable up to 50°C. This enzyme activated plasminogen and also degraded the fibrin net of blood clot, which suggested its potential as an effective thrombolytic agent.
Conclusions: Wheat bran was found to be an effective substrate for the production of fibrinolytic enzyme. The purified fibrinolytic enzyme degraded fibrin clot. The fibrinolytic enzyme could be useful to make as an effective thrombolytic agent.

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1. Introduction

Cardiovascular diseases (CVDs) such as acute myocardial infarction, ischemic heart diseases, and stroke are the leading cause of death worldwide, and the number of deaths due to CVDs is expected to increase over 23.3 million by 2030 [1]. The fibrinolytic agents such as urokinase plasminogen activator (u-PA), tissue plasminogen activator (t-PA), and streptokinase are commonly used to treat CVDs. However, the thrombolytic agents such as t-PA and u-PA are generally safe but are very expensive; later is a cheap fibrinolytic agent but causes undesirable side effects such as gastrointestinal bleeding [2]. Considering the global burden, the search continues for a safe and cheap thrombolytic agent to treat CVDs. In recent years, many studies on bacterial fibrinolytic enzymes have been proven as a safe thrombolytic agent. Likewise, Mine et al. [3] described the use of fibrinolytic enzymes from food-grade microorganisms. The bacterial strains such as *Bacillus subtilis* A26 [4], *Bacillus amyloliquefaciens* [5], *Paenibacillus* sp. IND8 [6], *Xenorhabdus indica* KB-3 [7], and *Bacillus* sp. [8] were used for the production of fibrinolytic enzymes in recent years. Bacterial fibrinolytic enzymes were produced by both submerged fermentation and solid-state fermentation (SSF).

SSF is a potential technology for the production of various biomolecules including enzymes. The cheap and easily available agro-industrial wastes such as apple pomace [9,10], rice chaff [11], pigeon pea [12], potato peel [13], and coffee pulp [14] were used as the sole source of carbon for the production of proteolytic enzymes in SSF. In SSF, the enzyme yield is relatively higher than submerged

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fermentation process. The main advantage of SSF is to utilize cheap agro-industrial wastes as the substrate for the production of enzymes. Statistical experimental design has many advantages over traditional one-at-a-time optimization strategy. The statistical tool such as fractional factorial design [15], Plackette-Burman's design [16], and L-18 orthogonal array [17] was frequently used in enzyme bioprocess. One of the important tasks in an enzyme bioprocess is the production of enzyme in cheaper cost. Hence, optimization of process parameters by statistical approach, such as two-level full factorial design and Response Surface Methodology (RSM), could improve the enzyme yield significantly and decreases the production cost of enzymes. The traditional one-at-a-time strategy method of optimization frequently failed to locate the optimum response. RSM has been frequently applied for the production of enzymes such as nattokinase [15], glucose oxidase [18], arginine deiminase [19], L-asparaginase [20], and laccase [21], and so on. The report on statistical optimization of fibrinolytic enzyme production in SSF is limited. The main aim of this study was to optimize the medium components by RSM to enhance fibrinolytic enzyme production in SSF using agro-industrial wastes and to study the properties of enzyme.

2. Materials and methods

2.1. Screening of fibrinolytic enzyme producing bacterial isolate

Rice was boiled in water for 45 min and was allowed for aerobic fermentation for 48 h at room temperature (30 ± 2°C). This fermented rice was used as the source of fibrinolytic enzyme producing bacteria. Ten grams of fermented rice was suspended in double distilled water and screened on skimmed milk agar plates for isolation of proteolytic bacteria. Thirteen bacterial isolates showed halo zone on skimmed milk agar plates were further cultured using wheat bran substrate in SSF. The pH of the substrate was adjusted as 9.0 using glycine-NaOH buffer (0.1 M), and 80% (v/w) moisture level were maintained. The substrate was autoclaved at 121°C for 15 min, and SSF was initiated by inoculating individual bacterial isolates (10%, v/w). These flasks were incubated at 37°C for 72 h, after which 50 mL double distilled water was added and placed in an orbital shaker for 30 min at 175 rpm. It was further centrifuged at 5000 × g for 20 min at 4°C, and the supernatant was used as the source of crude enzymes. Fibrinolytic activity of the crude enzyme of the bacterial isolates was tested individually using a fibrin plate composed of 1% (w/v) agarose, 0.5% (w/v) fibrinogen, and 50 μL (100 NIH U/mL) thrombin [22]. This plate was allowed to stand for 1 h at room temperature (30°C ± 2°C) to form a fibrin clot layer, and 10 μL of crude enzyme from the individual isolate was dropped into wells. The plates were incubated at 37°C for 5 h, and the fibrinolytic activity exhibited a clear zone of fibrin degradation around the well.

2.2. Identification of the bacterial isolate

In the present study, 10 bacterial isolates showed fibrinolytic activity, and the potent bacterial isolate was used for further studies. The potent bacterial isolate was identified using its morphological, biochemical characters [23] and 16S rRNA sequencing using forward (P1: 5′-AGAGTTTGATCMTGGCTAG-3′) and reverse primer (P2: 5′-ACGGGCGGTGTGTRC-3′). The DNA was amplified by using a Peltier Thermal Cycler Machine (USA) and DNA polymerase (Sigma-Aldrich, USA). The resulted PCR amplicon was sequenced and compared the sequence with the database using BLAST NCBI server [24]. The 898 bp 16S rDNA sequences of the bacterial isolate were submitted to GenBank.

2.3. Substrate

The agro-industrial wastes such as banana peel, black gram husk, cow dung, paddy straw rice bran, and wheat bran were collected from Nagercoil, Kanyakumari district, Tamilnadu, India. These substrates were dried individually under sunlight for a week and powdered using a mixer grinder. Finally, these substrates were stored in a container individually for further use.

2.4. Screening of various agro-industrial wastes for the production of fibrinolytic enzymes

5.0 g of processed substrate (banana peel, black gram husk, cow dung, paddy straw, rice bran, and wheat bran) was taken in a 100-mL Erlenmeyer flask individually. These substrates were moistened with glycine-NaOH buffer (pH 9.0, 0.1 M) at 80% (v/w) level. The contents were mixed thoroughly and autoclaved at 121°C for 20 min. The sterilized medium was inoculated with 18 h grown *Bacillus halodurans* IND18 at 10% (v/w) level and incubated for 72 h at 37°C. The fibrinolytic enzyme was extracted from agro-residues using 50 mL glycine-NaOH buffer (pH 9.0, 0.1 M) by shaking on a rotary shaker at 150 rpm for 30 min. The clear supernatant was used as the crude enzyme.

2.5. Assay of fibrinolytic activity

Fibrinolytic enzyme activity of the culture supernatant was carried out using fibrin substrate [25]. The reaction mixture contained 2.5 mL fibrin (1.2%, w/v), 2.5 mL Tris–HCl buffer (0.1 M, 0.01 M $CaCl_2$, pH 7.8), and 0.1 mL crude enzyme. It was incubated at 37°C for 30 min, and the reaction was stopped by the addition of 5 mL of 0.11 M trichloroacetic acid containing 0.22 M sodium acetate and 0.33 M acetic acid. The reaction mixture was centrifuged for 15 min at 10,000 × g, and the clear supernatant was read at 275 nm using a UV–visible spectrophotometer. One fibrinolytic unit was defined as the amount of enzyme that gave an increase in absorbency at 275 nm equivalent to 1 μg of tyrosine/min at 37°C.

2.6. Screening of process parameters by one variable-at-a-time strategy

In the present study, wheat bran was used as the substrate for optimization of enzyme production in SSF. The process parameters such as fermentation period (24–96 h), pH (6.0–10), inoculum (2–12%), moisture content (40–100%), carbon sources (1% maltose, sucrose, starch, glucose, and trehalose), nitrogen sources (1% yeast extract, peptone, beef extract, gelatine, and urea), and inorganic ions (0.01% ammonium sulphate, ammonium chloride, disodium hydrogen phosphate, sodium nitrate, sodium dihydrogen phosphate, calcium chloride, and dipotassium hydrogen phosphate) on the production of enzymes were studied.

2.7. Screening of significant factors by statistical approach

The medium constituents toward fibrinolytic enzyme production were screened by a full factorial experimental design. The significant physical parameters (pH and moisture) and the nutritional factors (glucose, peptone, and $CaCl_2$) were subjected for screening. The 2^5 factorial design consisted of 32 experimental runs in which the selected five factors such as pH, moisture, glucose, peptone, and $CaCl_2$ were kept either at their low (-) or high (+) levels. The other process parameters such as fermentation period and inoculum were kept at optimum level. The statistically designed culture media were prepared according to the designed protocol and inoculated with the seed culture at 10% level (v/w) and incubated at 37°C for 72 h. At the end of the fermentation period, fibrinolytic

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