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Short communication

Response surface methodology based optimization of keratinase production from alkali-treated feather waste and horn waste using *Bacillus* sp. MG-MASC-BT

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ABSTRACT

A native feather-degrading keratinolytic bacterium, *Bacillus* sp. MG-MASC-BT was isolated from feather dumping soil in Mallasamudram, Tamil Nadu, India and screened for keratinase production using alkali treated horn waste (HW) and feather waste (FW). The study factors influencing keratinase production was optimized by Box–Behnken design (BBD). The maximum enzyme production (1075 U/ml) was observed at pH 7.0, temperature 55 °C and growth period of 60 h. The media supplemented with 6% of HW and FW enhanced keratinase production. Statistics based contour plots were generated to evaluate the changes in the response surface and to understand the relationship between the enzyme yield and the culture conditions.

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Introduction

Proteases (EC 3.4.21–24) are a group of enzyme-hydrolyzed peptide bonds present in the proteins. Kertinase (EC 3.4.99.11), a protease enzyme, target mostly hydrophobic amino acid-rich, insoluble proteins. Thus, keratinase is easily differentiated from other proteases as they have >50% activity on insoluble keratin when compared with soluble proteins [1,2]. The enzyme has promising applications in leather, detergent, medicine, and cosmetics industries. In addition, keratinase has great interests in the enzyme-market owing to the fact that there is a great demand for developing biotechnological alternatives for recycling of keratin-wastes such as feather, hair, horn, hooves, nail, and scales, in environmental concern [1,3,4]. Different group of microorganisms such as bacteria and fungi have been investigated for the production of keratinase [5–13]. Among these, bacterial keratinases have received much attention in the industries. Recently, the bacteria *Bacillus* sp. is commonly employed for the production of several protease enzymes because of the rapid growth rate, capability to utilize several industrial wastes as carbon and nitrogen source, ability to grow in different ecological circumstances, and potential to secret different types of extracellular enzymes [14,15].

Solid state fermentation (SSF) is a biotechnological process in which fermentation reaction is carried out in solid matrix with limited amounts of moisture and substrate. The limited moisture enhances the growth and metabolic activity of the bacteria and thereby increases the maximum production of product of interest, and reduces the time required for downstream processing [16,17]. Several bacterial enzymes such as alpha amylase, cutinase, cellulase, protease, xylanase, and keratinase have been successfully produced in SSF using different groups of bacteria [18–21].

Response surface methodology (RSM) is a widely accepted modern statistical approach for optimization of experimental conditions and solving analysis of problems, in which a response is greatly influenced by several variables for the production of industrially important biomolecules. RSM aided to identify the successful factors, study interactions, most favorable conditions, calculate the optimum level of the variables, and ensure the

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maximum production in a fixed number of experiments [22,23]. In earlier studies, RSM based approach has been proved to be an effective statistical method to enhance enzyme production by SSF [24].

Animal feathers are the predominant waste material disposed by poultry based meat processing industries. Incremental increases of these industrial wastes all over the world have resulted in the generation of millions of tons of these wastes annually. Continuous discharging of these wastes without recycling leads to high pollution load in the ecosystem. Horn meal is disposed in large quantities from animal by-product processing industries. Horn wastes are reported as an attractive and inexpensive substrate for the production of keratinase enzyme [25]. Several studies reported the keratinase production with using single substrate such as feather [26,27] and horn meal [25,28]. However, keratinase production with bi-substrate combination has rarely been discussed, particularly on utilization of FW and HM. It has been established that the fermentation variables and strategies are highly influenced the production rate of keratinases. Hence, the objectives of the present study were (i) isolation, identification and screening of bacteria for keratinolytic activity; (ii) evaluate the potential of FW and HM as substrates for keratinase production under SSF; (iii) optimization of physico-chemical condition by BBD; (iv) assess the activity of enzyme at different pH and temperature.

Materials and methods

Isolation and screening of keratinase producing bacteria

Soil samples were collected from a feather disposal site of a poultry plant, Mallasamudram, Tamil Nadu, India. The samples were carefully transported to the laboratory and processed within 12 h. The cultivable bacteria were isolated by serially diluting soil sample (1 g) in sterile water, and 100 μl of the diluted sample were plated onto Nutrient Agar (Hi-Media, India) by spread plate technique [29]. Later, the plates were incubated at 28 °C for 48 h and observed for the bacterial growth. Morphologically different bacterial colonies were purified and aseptically inoculated onto Skim Milk Agar (Hi-Media, India) [30]. The plates were incubated at 28 °C for 24 h and observed for the clear zone around the bacterial colonies.

16S rDNA sequencing and phylogenetic analysis

Chromosomal DNA was extracted from the potential isolate MG-MASC-BT according to Maniatis et al. [31]. The partial 16S rRNA gene was amplified using universal 27f (5′-AGAGTTTGATCCTGGCTCAG-3′) and 907r (5′-CCCCGTCAATTCATTTGAGTTT-3′) primers. The amplicon was purified (QIAGEN, CA, USA) and sequenced in both forward and reverse directions using ABI PRISM (Model 3700, CA, USA). The sequences were compared using BLAST analysis (NCBI) in order to identify the potential isolate MG-MASC-BT. Phylogenetic analysis was performed using Neighbor-Joining method in CLC WORKBENCH 5.2 software (CLC bio, MA, USA).

Substrates preparation

The substrate feather waste (FW) was procured from poultry industry and raw horn waste (HW) was collected from local slaughter house, Namakkal, Tamil Nadu, India. The procured substrates were processed according to Rai et al. [18] and Karthikeyan et al. [32]. Later, substrates were subjected to alkaline treatment using 1% (w/v) NaOH. Briefly, 5 g of substrate was dispensed in 100 ml of alkali solution and left at room temperature for 2 h. The alkali treated substrates were washed with distilled water until the filtrate become neutral and dried at 37 °C for 24 h, and used for the fermentation studies [33].

Scanning electron microscopy

Scanning electron microscopy (SEM) was used to examine morphological modifications of raw and alkali pretreated substrates. Samples were dehydrated and mounted on aluminum stubs and sputter-coated with gold for 300 s using high vacuum and a voltage acceleration of 10 kV. The SEM was performed in a JEOL JSM 6390 model.

Solid state fermentation and statistical optimization

The substrates were vigorously mixed in 500 ml Erlenmayer flask and autoclaved at 121 °C for 15 min. After sterilization the flasks were cooled and inoculated with 5 ml of isolate MG-MASC-BT (10^8 cells/ml (0.8 OD at 600 nm)) under aseptic condition.

Response surface methodology based Box Behnken design was used for the statistical optimization of experimental conditions. Four independent variables such as incubation time, temperature, FW, and HW were selected for the study and the experiments were designed using design expert software (9.0 trial version). Totally 29 experiments were executed to optimize the process parameters according to the design. Keratinase activity was determined by coefficient of determination (R^2), analysis of variance (ANOVA) and contour plots. Employing RSM, the most widely used second-order polynomial equation developed to fit the experimental results and identify the relevant model terms

$$Y = \beta_0 + \sum \beta_i X_i + \sum \beta_{ii} X_i^2 + \sum \beta_{ij} X_i X_j \quad (1)$$

where Y is the predicted response; β_0, β_i, β_{ii}, β_{ij} are fixed regression coefficients of the model; X_i and X_j represents independent variables.

Keratinase assay

Fermented solution was recovered from the flask, centrifuged at 7000 rpm for 10 min at 4 °C and the supernatant was used for keratinase enzyme assay according to Sahoo et al. [34] with minor modifications. Briefly, 0.2 ml of the crude keratinase was mixed with 0.3 ml of keratin solution, followed by 0.2 ml of 100 mM Tris–HCl buffer (pH 8.0) and incubated at 55 °C for 15 min. The reaction was terminated by the addition of 0.1 ml of 10% trichloroacetic acid TCA. After 10 min incubation the reaction mixture was centrifuged at 10,000 rpm for 15 min and the keratinase concentration was analyzed using UV spectrophotometer at the wavelength 280 nm. One unit (U) of keratinase activity was measured as an increase of corrected A_{280} by 0.01 under the assay conditions.

Effect of pH and temperature on the activity and stability

The pH (6–11) stability of keratinase was determined using 50 mM phosphate buffer (pH 7.0, 8.0), Tris–HCl (pH 7.0–9.0), glycine–NaOH/sodium carbonate–bicarbonate buffer (pH 9.0–11.0) and glycine–phosphate–NaOH buffer (pH 10.0, 11.0). The thermal stability of the enzyme was studied in the range 30–80 °C under optimum conditions.

Results and discussions

In the present study, an effort has been made to maximize the keratinase production under optimized condition using two different substrates in SSF. Four morphologically similar bacterial colonies were isolated from the soil and subsequently screened

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