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Optimization of thermotolerant alkaline protease production from *Brevibacillus brevis* strain BT2 using surface response methodology

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ABSTRACT

An efficient thermotolerant alkaline protease producer, strain *Brevibacillus brevis* BT2 was isolated from waste water effluents from textile dyeing industry. Effect of variables, i.e. soybean meal, soluble starch, inoculums, temperature and pH and their mutual interaction were analyzed by Box-Behnken design (BBD) for optimizing their levels in the medium and effect of their mutual interaction on thermotolerant alkaline protease production. All the above variables were found significantly influencing protease activity individually and also in interaction with other variables. The model predicted maximal protease production of 121.1 ($U\,ml^{-1}$) at the soybean meal 7.46 (gL^{-1}), soluble starch 10.00 (gL^{-1}), inoculums 1.76%, temperature 49.3 °C, and pH 9.1. The maximum protease production obtained experimentally was found to be 128.5 U/ml. The result was coincident with the predicted value and the model was proven to be adequate.

1. Introduction

Enzymes have long been used as alternatives to chemicals in many industrial systems (Illanes, 2008) and their demand is continuously rising as evident through their estimated market potential of $4.4 billion in 2015, and is expected to further reach about $6 billion by 2018. Proteases are one of the most important industrial enzymes and are used in a various of industrial processes such as laundry detergents, leather industry in dehairing and bating of hides, preparation of protein hydrolysate, coagulation of milk for cheese production, and pharmaceutical industry, therapeutic applications preparations and. constitute over 60% of total enzyme market (Li et al., 2013; Adrio and Demain, 2014). Microbial proteases are preferred to enzymes from plant and animal sources since they possess almost all the characteristics desired for biotechnological applications (Rao and. Narasu, 2007). About 35% of the total microbial enzymes used in detergent industry are derived from bacterial sources and most of them produced by *Bacillus* sp. and can be cultivated under extreme temperature and pH conditions to give rise to products that are, in turn, stable in a wide range of harsh environments (Ferrero et al., 1996).

The major obstacle in large-scale production of alkaline protease is a high cost of growth media. With the increasing emphasis on cost effectiveness for enzyme production, the use of low-cost fermentation medium and process optimization is of paramount importance for commercial gain. Recently, only a few studies investigated the protease producing potential of the *Brevibacillus brevis* (Jaouadi et al., 2013; Olajuyigbe et al., 2014) but there is no previous report on process optimization using the design of experiment (DOE). In view of this, a statistical experimental design was employed to optimize culture conditions for higher alkaline protease using *Brevibacillus brevis* BT2 strain under submerged fermentation. A response surface methodology combined with Box-Behnken design (BBD) was then used to further optimize the effect of variables including soybean meal, soluble starch, inoculums, temperature, and pH.

2. Materials and methods

2.1. Isolation thermotolerant protease producing bacteria

The thermophilic bacteria were isolated from dye contaminated hot water effluent from textile dyer in Dehradun, Uttarakhand. An aliquot of 100 µL of sample was cultivated in 15 ml of MSM medium on a gyrorotatory shaker for 24 h at 55 °C. Subsequently, the isolates capable of growing at this temperature were further screened for proteolytic protease activity by streaking them on skim milk agar plates (pH 7.5) and incubated at 55 °C for 48 h. Individual colonies that produced clear zones were purified *via* subculturing. Proteolytic Index (IP) was determined by comparing the diameter of the clear zone and colony growth diameter of the growth zone. The bacterial isolate with higher IP was selected and one of the isolates producing the largest hydrolysis

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zone was selected and designated as BT-2. The strain was then maintained in 40% glycerol solution and stored at −80 °C until further use.

2.2. Identification of proteolytic strain and phylogenetic analysis

For identification of proteolytic strain BT2 through 16S rDNA sequence analysis, g-DNA was isolated from the using a fast DNA kit (Q-Biogene). In the PCR reaction, 16S rDNA was amplified using universal primer sets: 27F (5′-AGAGTTTGAT CCTGGCTCAG-3′), and 1492R (5′-GGTTACCTTGTTACGACTT-3′). PCR amplification was performed in a Thermal Cycler (Thermo Fisher Scientific, Waltham, MA, USA) and experimental conditions were as follows: 50 μL reaction mixture containing 100 ng of total DNA, 2U of Taq polymerase, 0.2 mM of dNTPs 3.0 mM of $MgCl_2$ and 0.4 μM of each primer. The PCR amplification was carried out using the initial denaturation step of 10 min at 94 °C, followed by 30 cycles 1 min 94 °C, 30 s 55 °C, 72 °C for 1 min The reaction was completed at final extension temperature 72 °C for 10 min The presence of PCR products was determined by electrophoresis of 10 μL of the reaction product in a 1.5% agarose gel. The purified PCR products were sequenced using ABI Big Dye Terminator chemistry v3.0 (Applied Biosystems). The 16S rRNA gene sequence of the isolate BT2 was deposited in GeneBank using BankIt submission tool and obtained accession number KM216316. The 16SrRNA sequence was aligned and compared with other 16SrRNA genes in the GenBank by using the NCBI BLASTn program. A distance matrix was generated using the Jukes-Cantor corrected distance model. The phylogenetic trees created using NJ (Neighbor Joining) method.

2.3. Thermotolerant protease production in shake-flask culture

For routine culture, a loop full of pure culture was inoculated into 50 ml of sterile nutrient broth. An aliquot of 1 ml actively growing culture ($A_{550\ nm}$=0.250–0.300) was inoculated in 50 ml production medium taken in 250 ml Erlenmeyer flasks containing (g/L): Glucose 1.0, peptone 5.0, yeast extract 0.2, $CaCl_2$ 0.1, K_2HPO_4 0.2, $MgSO_4 0.7h_2O$ 0.1. The inoculated medium was incubated in a gyrorotatory shaker at 37 °C with agitation speed 200 rpm. The culture was centrifuged at 10.000 *g* for 10 min at 4 °C, the cell pellet was discarded and the supernatant was used for the assay of protease activity.

2.4. Determination of protease activity

The protease was assayed using casein as substrate. A 0.5 ml aliquot of the culture supernatant, suitably diluted, was mixed with Tris–HCl buffer (0.01 M, pH 9.0) containing 1% casein, and incubated for 15 min at 50 °C. The reaction was stopped by addition of 0.5 ml 20% trichloroacetic acid. The mixture was allowed to stand at room temperature for 15 min and then centrifuged at 10,000 *g* for 15 min to remove the precipitate. The absorbance of the supernatant was measured at 280 nm. A standard curve was generated using solutions of 0–50 mg/l tyrosine. One unit of the protease was equivalent to the amount of enzyme required to release 1 μg of tyrosine/ml/min under standard assay conditions. The relative protease activity was defined as the percentage of activity detected with respect to the maximum protease activity detected in the enzymatic assay.

2.5. Screening of carbon and nitrogen sources by one-factor-at-a-time (OFAT) design

Screening of media components for the production of the protease was done initially using one- variable-at-a time strategy.

Table 1.
Levels of variables used in the experimental design.

Variables	Range and levels		
	−1	0	+1
X_1: Soyabean meal (g L^{-1})	5	7.5	10
X_2: Soluble starch (g L^{-1})	10	12.5	15
X_3: Inoculum (%)	1	1.5	2
X_4: Temperature (°C)	37	46	55
X_5: pH	7	8.5	10

The different carbon sources including soluble starch, sucrose, maltose, glucose, and fructose were added to production medium at a final concentration of 1% carbon (w/v). To study the effect of different nitrogen sources on protease production, soybean meal, yeast extract, peptone, tryptone and ammonium sulfate, was separately added to nutrient medium at a final concentration of 0.5% nitrogen (w/v). The medium component that supports the best protease activity was undertaken for further experiment on optimization of protease production.

2.6. Optimization of medium components and fermentation condition for alkaline protease production

The optimal levels of culture conditions (Soybean meal, soluble starch, pH, temperature and inoculums (%) and incubation time), and the interactions were analyzed by Box-Behnken design of response surface methodology (RSM). Experimental range and levels of the five independent variables used in Box-Behnken design in terms of actual and coded factors has been shown in Table 1. In this study, five independent variables, three levels BBD with 46 runs were employed (Table 2).

The response data obtained from BBD design experiment on protease production was fitted to the following second-order polynomial Eq. (1).

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

where *Y* is the predicted response, β_0 is the intercept, x_i and x_j are coded independent variables, β_i is the linear coefficients, β_{ii} is the quadratic coefficients, and β_{ij} is the interaction coefficients, ε random error. The statistical model was validated with respect to all the three variables within the design space. A random set of four experiments was used to verify protease production under the experimental conditions.

2.7. Partial purification of protease

After validation of RSM, the protease production was undertaken in the optimal medium. Five percent inoculum from overnight grown culture of *Brevibacillus brevis* was added in freshly prepared above medium. One litre of the cell-free supernatant was collected from the 48 h grown culture by centrifugation at 10,000 rpm for 10 min at 4 °C. The supernatant was subjected to 45% ammonium sulfate saturation. Then, the solution was centrifuged at 12,000 *g*, 4 °C for 30 min and the precipitate containing enzyme was collected, and the pellet was resuspending in 20 mM phosphate buffer (pH 7.0). Subsequently, the suspension was dialyzed thoroughly against the same buffer for desalting.

2.8. Effect of pH and temperature

To determine the optimum pH, enzyme activity was assayed using casein as substrate at the pH range of 4.0–11.0 at 50 °C. The