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Optimization of protease production by *Microbacterium* sp. in feather meal using response surface methodology

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Abstract

A 2^3 factorial design was performed with the aim of optimizing protease production by a strain of *Microbacterium* sp. isolated from feathers in decomposition by response surface methodology. Protease production was first tested on different nitrogen source (casein, peptone, yeast extract, gelatin, soybean protein, feather meal and cheese whey). Feather meal was the selected substrate to test the effect of three variables on protease production (temperature, initial pH and feather meal concentration) by RSM. The point was chosen with these conditions: temperature 37 °C, initial pH 7.0 and feather meal concentration 12.5 g l^{-1}. Statistical analysis of results showed that, in the range studied, only pH did not have a significant effect on protease production whereas interaction between pH and feather meal concentration was significant. The optimum conditions were 25 °C, initial pH 7.0 and 12.5 g l^{-1} of feather meal. Under these conditions, the model predicted a protease activity of 202.7 U ml^{-1}.

Keywords: Actinomycetes; *Microbacterium*; Protease; Experimental design; Optimization

1. Introduction

Proteases constitute at least 65% of the total industrial enzyme market [1]. They are used for various industrial applications, such as laundry detergents, leather preparation, protein recovery or solubilization and organic synthesis [2]. In food industry, proteases have been routinely used for various purposes such as cheesemaking, baking, preparation of soy hydrolysates and meat tenderization [1].

Bacterial neutral proteases are active within a narrow pH range and have relatively low thermal tolerance. Due to their intermediate rate of reaction, neutral proteases generate less bitterness in hydrolyzed food proteins than do the animal proteinases and hence are valuable for use in the food industry [3]. Their low thermal tolerance is advantageous for controlling their reactivity during the production of food hydrolysates with a low degree of hydrolysis [1].

Optimization of medium by the classical method involves changing one independent variable (i.e., nutrient, pH, temperature) while unchanging all others at a fixed level. This is extremely time-consuming and expensive for a large number of variables [4] and also may result in wrong conclusions [5]. Response surface methodology (RSM) is a collection of statistical techniques for designing experiments, building models, evaluating the effects of factors and searching optimum conditions of factors for desirable responses [6]. This method has been successfully applied in many areas of biotechnology such as bioconversion of cheese whey to mycelia of *Ganoderma lucidum* [7], optimization of neomycin production [4], enzyme production [8], enzyme kinetics [9] and bacteriocin production [10]. With respect to protease production, it was utilized for example for *Bacillus* species [11,12]. Extracellular protease production by microorganisms is greatly influenced by physical factors such as pH, temperature and incubation time and by others factors such as media composition and presence of metal ions [13–16].

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The feather-degrading *Microbacterium* sp. strain kr10 produces an extracellular keratinase belonging to the metalloprotease family. This enzyme was also proved to present desirable properties for de-hairing bovine pelts, which would be very beneficial to the leather industry [17]. The purpose of this study was to evaluate the effect of three variables (temperature, pH and feather meal concentration) in the production of this protease. Previously, enzyme production with different by-products as growth substrate was tested.

2. Materials and methods

2.1. Reagents and media

Azocasein was from Sigma Chemical Co. (St. Louis, USA). Feather meal and soybean protein were from Bunge Alimentos S.A. (Brazil). Dried cheese whey powder was from Parmalat (Porto Alegre, Brazil). Nutrient agar and peptone were from Oxoid (Basingstone, UK) and yeast extract was from Biobras (Montes Claros, Brazil). Casein and gelatin were from Synth (Diadema, Brazil).

2.2. Bacterial strain and inoculum preparation

Microbacterium sp. strain kr10 was used as the producer microorganism. BHI medium containing 20% (v/v) glycerol was used for maintenance of the strain at −20 °C. The cells were first propagated in milk agar plates (5 g l^{-1} peptone, 3 g l^{-1} yeast extract, 12.0 g l^{-1} agar and 100 ml l^{-1} UHT skimmed milk). The inoculum was prepared in feather meal previously hydrolyzed with 0.4N NaOH for 2 h, neutralized with HCl, filtered and than autoclaved (121 °C for 40 min). The inoculum absorbance at 600 nm was adjusted before use with a Hitachi U-1100 spectrophotometer (Hitachi, Japan) to obtain about 1×10^6 CFU ml^{-1}.

2.3. Selection of nitrogen source

For selection of the best nitrogen source for protease production, various inorganic and complex nitrogen sources (casein, peptone, yeast extract, gelatin, soybean protein, feather meal and cheese whey) were tested individually (10 g l^{-1}) in the minimal mineral medium (0.5 g l^{-1} NaCl, 0.3 g l^{-1} K_2HPO_4, 0.4 g l^{-1} KH_2PO_4, 0.015 g l^{-1} $CaCl_2{\cdot}2H_2O$). Erlenmeyer flasks of 200 ml containing 50 ml of medium were inoculated with 1% of inoculum ($A_{600\ nm}$ at 0.350) and incubated at 30 °C under shaking (125 rpm). Protease yield was determined after 48 h.

2.4. Experimental design and protease production

After selection of the best medium, the next stage was determination of the optimal levels of three variables, feather meal concentration, temperature and initial pH on protease production. For this purpose, the response surface approach by using a set of experimental design (central composite design with five coded levels) was performed. For the three factors, this design was made up of a full 2^3 factorial design with its eight points augmented with three replications of the centre points (all factors at level 0) and the six star points, that is, points having for one factor an axial distance to the centre of $\pm\alpha$, whereas the other two factors are at level 0. The axial distance α was chosen to be 1.68 to make this design orthogonal. A set of 17 experiments was carried out. The range and levels of experimental variables investigated are presented in Table 1. The central values (zero level) chosen for experimental design were: feather meal concentration 12.5 g l^{-1}, temperature 37 °C and initial pH 7.0. The enzyme activity values were tested after 2, 4 and 6 days of incubation but the regression equation was made with the values obtained after 4 days (maximal values). In developing the regression equation, the test factors were coded according to the following equation:

Table 1
Values of independent variables at different levels of the 2^3 factorial design

Independent variables	Symbol	Levels				
		−1.68	−1	0	+1	+1.68
Initial pH	x_1	5.0	5.8	7.0	8.29	9.0
Temperature (°C)	x_2	25	30	37	45	50
Feather meal (g l^{-1})	x_3	0.0	5.0	12.5	20.0	25.0

$$x_i = \frac{X_i - X_0}{\Delta X_i} \quad (1)$$

where x_i is the coded value of the ith independent variable, X_i the natural value of the ith independent variable, X_0 the natural value of the ith independent variable at the center point and ΔX_i the step change value (ΔX_i is 1.2 for initial pH, 8 for temperature and 7.5 for feather meal concentration). For a three factors system, the model equation is:

$$Y = b_0 + b_1x_1 + b_2x_2 + b_3x_3 + b_{11}x_1^2 + b_{22}x_2^2 + b_{33}x_3^2 + b_{12}x_1x_2 + b_{13}x_1x_3 + b_{23}x_2x_3 \quad (2)$$

where Y, predicted response, b_0, intercept; b_1, b_2, b_3, linear coefficients; b_{11}, b_{22}, b_{33}, squared coefficients; b_{12}, b_{13}, b_{23}, interaction coefficients.

Results were analyzed by the Experimental Design Module of the Statistic 5.0 software (Statsoft, USA). The model permitted evaluation of the effects of linear, quadratic and interactive terms of the independent variables on the dependent variable. The statistical significance of the regression coefficients was determined by Student's t-test and the second order model equation was determined by Fisher's test. Three-dimensional surface plots were drawn to illustrate the main and interactive effects of the independent variables on protease production. The optimum values of the selected variables were obtained by solving the regression