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Statistical optimization of L-leucine amino peptidase production from *Streptomyces gedanensis* IFO 13427 under submerged fermentation using response surface methodology

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ABSTRACT

Response surface methodology (RSM) employing the central composite design (CCD) was used to optimize the fermentation medium for the production of L-leucine amino peptidase (LAP) from *Streptomyces gedanensis* IFO13427 under submerged fermentation. The design was employed by selecting substrate concentration, NaCl concentration and initial pH as model factors by 'one variable at a time' experiment. A second-order quadratic model and response surface method showed that the optimum conditions (soy bean 0.3%, NaCl, 0.03 M, and initial pH 7) resulted in the improvement of LAP production (25.69 IU/ml) as compared to the initial level (12.17 ± 0.23 IU/ml) after 72 h of fermentation, whereas its value predicted by the quadratic model was 24.56 IU/ml. Analysis of variance (ANOVA) showed a high coefficient of determination (R^2) value of 0.9799, ensuring a satisfactory adjustment of the quadratic model with the experimental data. This is first report on LAP production by *S. gedanensis* using statistical experimental design and response surface methodology in submerged fermentation.

1. Introduction

Amino peptidases (aminoacyl-peptide hydrolase, E.C 3.11) constitute a diverse set of peptidases with important roles in cell maintenance, growth and development, and defense. These enzymes have a broad substrate specificity that catalyze the removal of amino acids from the N-terminus of a protein and are widely distributed in many tissues and cells in plants, animals, bacteria and viruses [1]. Leucine amino peptidase (LAP, EC 3.4.11.1) is a widely distributed metallo exopeptidase that catalyzes the hydrolysis of leucine residues from the amino-termini of protein or peptide substrates [2]. It has been the most extensively studied enzymes because it plays key role in the metabolism of biologically active peptides [3,4] and has great potential for commercial applications [5,6]. The intensity of the bitterness for a peptide is proportional to the number of hydrophobic amino acids [7]. L-Leucine amino peptidase (LAP) can remove single or pairs of hydrophobic amino acids from the N-terminus of a polypeptide chain, it is considered to be useful for debittering protein hydrolysates with N-terminal hydrophobic amino acids, which are commonly used as clinical nutrition supplements [6,8]. It can be used to improve flavor development [9] and for dipeptide synthesis [10] Commercial LAPs are used to reduce or eliminate bitterness are from *Lactobacillus lactis*, *Rhizopus oryzae*, *Aspergillus sojae*, and *A. oryzae* [11].

Streptomycetes possess an intracellular proteolytic system composed of endopeptidases and exopeptidases, which cleave proteins for nutrition, remove aberrant and unwanted proteins, and also provide limited proteolysis for maturation and activation of newly synthesized polypeptide molecules. Streptomyces amino peptidases are of particular interest for biochemical, biomedical applications and also valuable for the preparation of a debittered protein hydrolysate in the food industry [12] because they are stable and have a low molecular weight, simple kinetics, high enzyme activity and broad substrate specificity [13]. In general, the leucine amino peptidases produced by *Streptomyces spp*. belong to the Zn2+ metallo-amino petidase group, whose activity is regulated by the presence of divalent metallic cations [14]. The majority of the amino peptidases purified from culture media of various *Streptomyces* species are leucine amino peptidases, including those from *S. fradiae* [15], *S. rimosus* [16], *S. griseus* [17,18], *S. lividans* [19], and *S. albidoflavus* [20]. Among different streptomyces strains screened, *Streptomyces mobaraensis* NRRL B-3729, *Streptomyces gedanensis* IFO 13427, and *S. platensis* NRRL 2364 were found to be good producers of extracellular LAP [21].

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In fermentation, the improvement in productivity of the microbial metabolite is achieved by manipulating the nutritional and physical parameters [22] and these can significantly alter the product yield.

Optimization of fermentation has long been used in enhancing the yield of many bioprocesses. The classical method of 'one variable at a time' approach permits the determination of specific requirements for growth and product formation by systematically adding or deleting components from the medium, with minimal complicated medium interactions [23]. Response surface methodology (RSM), which has been extensively applied in optimization of medium composition, conditions of enzymatic hydrolysis, fermentation, and food manufacturing processes [24], is the collection of mathematical and statistical techniques for experiment design, model development, evaluation factors, and optimum conditions of different biotechnological bioprocess. Statistical optimization not only allows quick screening of large experimental domain, but also reflects the role of each of the components. Optimization through factorial design and the application of response surface methodology is a common practice in biotechnology for optimizing the media components and process parameters [25,26]. However, no investigations have yet been carried out on the statistical optimization of media composition and culture conditions for leucine amino peptidase production from *S. gedanensis*.

The present study dealt with optimization of the most important fermentation variables using RSM for enhancing the LAP production by *S. gedanensis*. In the first optimization step 'one variable at a time' experiment was used to determine the likely effect of medium components on LAP production. In the second step, the factors that had significant effects were optimized using a central composite design (CCD) and response surface analysis. Three variables, substrate concentration, NaCl concentration and initial pH, were selected as the process (independent) variables while LAP production was the response (dependent) variable.

2. Materials and methods

2.1. Materials

L-Leucine-*p*-nitroanilide and 4-nitroaniline were procured from sigma chemicals (St. Louis, MO, USA). Agar and other medium components were purchased from Hi-media (Mumbai, India). All other analytical grade reagents were supplied by Merck (Mumbai, India) and SD Fine chemicals (Mumbai, India). Enzyme assay was performed by using UV160A spectrophotometer (Shimadzu Kyoto, Japan).

2.2. Microorganism and maintenance

S. gedanensis IFO 13427 was obtained from the Institute for Fermentation (IFO, Osaka, Japan). The organisms were grown and maintained at 30 °C in Petri plates made of ATCC-5 medium containing (gl^{-1}): Beef extract, 1; Yeast extract, 1; Trypton, 2; Glucose, 10; $FeSO_4{\cdot}7H_2O$, 0.1 and agar, 20; at pH 6.5 at 30 °C.

2.3. Inoculum preparation

S. gedanensis IFO 13427 was grown on ATCC-5 agar plates at 28 °C for 5 days for complete sporulation. A loop full of the spores were transferred to the inoculum medium (50 ml in 250 ml Erlenmeyer flask), which contained (gl^{-1}); Beef extract, 1; Yeast extract, 1; Trypton, 2; Glucose, 10; $FeSO_4{\cdot}7H_2O$, 0.1 at pH 6.0. The flasks were incubated at 30 °C and 200 rpm for 48 h. The inoculum contained 1.2×10^9 CFU/ml.

2.4. Submerged fermentation

Three fermentation media (Table 1) were used to evaluate the production of LAP in submerged fermentation by *S. gedanensis* IFO 13427. Unless otherwise specified, submerged fermentation was carried out in 250 ml Erlenmeyer flasks containing 100 ml medium, autoclaved at 121.5 °C for 20 min, inoculated with 2% (1.2×10^9 CFU/ml) inoculum and incubated at 30 °C under shaking (200 rpm) conditions for 5 days. Samples (whole flasks in triplicate) were collected at regular intervals and the contents were centrifuged at $7000 \times g$ at 4 °C for 15 min. The culture supernatant was used as the crude enzyme. Results reported are the average values with standard deviations.

2.5. Analytical methods

LAP activity assay in the culture supernatant was determined by the method of Tan and Konings [27], with slight modifications. The reaction mixture contained 1 ml of 2.5 mmol l^{-1} of L-leucine-*p*-nitroanilide (in 100 mmol l^{-1} NaOH–glycine buffer, pH 8.5), 1 ml 100 mmol l^{-1} of NaOH–Glycine buffer, pH 8.5 and 0.5 ml of the properly diluted supernatant. The well-mixed solution was incubated at 50 °C for 10 min. The reaction was stopped by the addition of 1 ml of glacial acetic acid and the absorbance was measured at 405 nm. Assay was carried out using appropriate substrate and enzyme blanks also. One International Unit of enzyme activity was defined as the amount of enzyme that hydrolysed 1 μmol of leucine-*p*-nitroanilide per minute. Standard plot was prepared with *p*-nitroaniline. The protein concentration was measured by the method of Lowry et al. [28], with crystalline bovine albumin as the standard.

2.6. Experimental designs and data analysis

The experiments were conducted to evaluate the effect of incubation temperature on LAP production for which fermentation was carried out at 25, 28, 30 and 35 °C shown in Table 2. To study the influence of initial pH of the medium on LAP production, the initial pH of the medium was set at 5.0–11.0 using 1 N NaOH or HCl. The effect of the inoculum on leucine amino peptidase production was studied by adding different concentrations (1, 2, 3, 4 ml) of 48 h old inoculum (1.2×10^9 CFU/ml). In order to select a natural feed-stock as the substrate, nine different substrates were procured from the local market and used at 0.5% concentration for fermentation to produce the LAP in submerged fermentation. Subsequently, different concentrations of the best substrate (0.125–5% w/v) were used to optimize the substrate concentration. In another set of the experiments, sucrose in the basal medium was replaced with 1% of different carbon sources, such as soluble starch, cellulose, inulin, carboxyl methyl cellulose (CMC), D-sorbitol, galactose, maltose, lactose and glucose. Studies were also carried out to evaluate the influence of different complex nutritive sources, such as beef extract, yeast extract, trypton, casein and urea by supplementing them 0.25% for the production of leucine amino peptidase. To evaluate the effect of inorganic nitrogen sources, 0.1% of various inorganic nitrogen sources, such as ammonium bicarbonate (NH_4HCO_3), ammonium phosphate (NH_4HPO_4), sodium nitrate ($NaNO_3$), ammonium nitrate (NH_4NO_3), ammonium sulphate ($(NH_4)_2SO_4$) and ammonium chloride (NH_4Cl) were added in the fermentation medium. The effect of supplementation of different non-ionic surfactants (Triton X-100, Tween 20, Tween 40, Tween 60 and Tween 80) on LAP production was studied by supplementing these in fermentation medium at 0.05%. To study the effect of inorganic salts on LAP production, medium was supplemented with 0.05% of $CaCO_3$, $NaHCO_3$, $CaCl_2$, $LiCO_3$, $Ca(OH)_2$, and