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THE PRODUCTION OF DETERGENT STABLE PROTEASES FROM *ASPERGILLUS NIGER* VIA OPTIMIZED SOLID STATE FERMENTATION

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Abstract

Proteases that are detergent stable are of great industrial value, because they are used in many laundry detergents and biotechnological processes. This research aimed to effectively create a protease enzyme that is compatible with detergents by Aspergillus niger by solid state fermentation (SSF) and optimize the production condition by Response Surface Methodology (RSM) to overcome the problem of high cost of producing the enzyme from agro-industrial residues. Substrate screening, optimization of five physico-chemical parameters (pH, temperature, inoculum size, incubation period, moisture content) by a Central Composite Design(CCD), extraction and partial purification of enzymes, biochemical characterization and compatibility testing of enzymes with commercial detergents were followed. The following parameters were used for the evaluation: protease activity (U/g), protein content, specific activity (U/mg) and statistical validation (ANOVA and regression modelling). Wheat bran was determined to be the most appropriate substrate leading to the highest protease activity (168.4 ± 4.2 U/g) and the RSM optimization increased the protease activity to ~305 U/g. The purified protease showed optimal activity at pH 4.0-4.5 and 40 °C and showed good stability of detergents and good shelf life performance of 30 days. This work is relevant for sustainable enzyme biotechnology by demonstrating the feasibility of the production of the protease in low-cost substrates that is not affected by the presence of detergent. Scale-up strategies and genetic engineering to further increase yield and stability may be explored in the future.

1. INTRODUCTION

Enzymes are biological catalysts that speed up chemical reactions under modest physiological conditions, making them useful in both natural and industrial processes. Proteases, a prominent family of enzymes, break peptide bonds in proteins; hence, proteins are made up of many proteases, with proteases being one of the most numerous and commercially significant enzyme families. Proteases make up more than 60% of the global enzyme market, highlighting their extensive use in industries such as food processing, pharmaceuticals, leather, and detergents. These adaptable enzymes can break down complex protein substrates into simpler peptides and amino acids, enabling their application in various biochemical and industrial processes.

In addition to recombinant production, proteases are being engineered for other environmental uses, such as wastewater treatment. In 2024, a study showed the immobilization of metagenome-derived proteases in periodic mesoporous organosilica frameworks (PMOFs), which greatly enhanced the efficiencies of protein hydrolysis in wastewater. The discovery opens the door to new applications of proteases, beyond their traditional

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uses in detergents, leather production, and pharmaceuticals, in sustainable environmental management. Immobilization technologies reduce environmental impact and costs, and bring increased enzyme stability and reusability.

The production of protease depends upon different parameters. The maximum production of protease having high activity under following optimized conditions like Effect of temperature, Effect of PH, Effect of Moisture content, Effect of Substrate concentration, Effect of Temperature and Effect of Inoculum size and concentration. It plays a decisive role in the production and activity of proteases, particularly alkaline proteases. These enzymes generally exhibit maximum activity in the range of 50–70 °C, which aligns with the metabolic capabilities of many thermos tolerant microorganisms. For instance, species such as Micrococcus sp. (50 °C), Bacillus clausii I-52 (60 °C), and Pseudomonas aeruginosa MN1 (60 °C) thrive within this range, producing proteases that remain stable and effective under elevated temperatures. Similarly, fungal strains like Aspergillus oryzae CH93 (50 °C) and actinomycetes such as Actinomycete MA1-1 (50 °C) contribute to industrial enzyme production by maintaining activity at moderately high temperatures, which is advantageous for large-scale processes.

One of the most important strengths of Aspergillus lies in its efficiency and adaptability. Lipases from Aspergillus species, for example, demonstrate high stability and broad substrate specificity, enabling their use in both hydrolytic and synthetic reactions. Similarly, proteases and amylases derived from these fungi are widely applied in food fermentation, textile processing, and biofuel production. This broad enzymatic capability underscores the versatility of Aspergillus as a biocatalyst, while advances in genetic engineering further enhance its industrial potential.

2.1 Material method

In the present investigation, the experimental study has been designed to prepare solid-state fermentation (SSF) protease of Aspergillus niger which is stable in the presence of detergent and optimized conditions by Response Surface Methodology (RSM). The overall workflow involves seven sequential stages: sourcing and preparation of agro-industrial substrates; procurement, maintenance and preparation of A. niger inoculum; preliminary screening of substrates for the production of protease; optimization of five physicochemical variables (pH, temperature, inoculum size, incubation period and moisture content) using RSM , harvesting and partial purification of crude enzyme, determination of protease activity and protein content, biochemical characterization and evaluation, compatibility study with commercial detergent brands and shelf-life test.

All the investigation is conducted in the Postgraduate Research Laboratory, Department of Biochemistry, Faculty of Engineering and Applied Sciences, Riphah International University, Islamabad, in a controlled laboratory environment.

Each experiment was repeated triplicate manner (n = 3) and results were reported as mean ± SD. The chemicals and reagents used in the study are analytical reagent grade and are purchased from reliable sources such as Sigma-Aldrich, Merck and Fluka. All glasswares were cleaned with chromic acid, then washed with distilled water and dried in the oven for use. All instruments used in the study, such as analytical balance, pH meter, autoclave, incubator shaker, UV–Visible spectrophotometer, refrigerated centrifuge, and rotatory evaporator were calibrated before each experimental run to ensure the reliability of the data.

The following well-known factors were considered in determining the production strain, Aspergillus niger, and the production platform, solid-state fermentation. A. niger is a GRAS (Generally Recognized As Safe) organism with a long history of safe use as an industrial enzyme producer for over 50 years and is well documented for its ability to produce a wide range of hydrolytic enzymes such as proteases, amylases, cellulases and pectinases. Secondly, the microbial proteases produced by the mesophilic fungi of the Aspergillus genus contribute more than 25% of the world production of microbial proteases, and in particular, A. niger is a strong competitor as it

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is one of the fungi that grows strongly in inexpensive lingo cellulosic substrates. Third, fungal enzyme production via solid-state fermentation presents several benefits compared to submerged fermentation: lower energy and water consumption, lower volume of liquid effluent, and often greater enzyme productivities per volume since filamentous fungi are naturally grown on solid matrices. Fourth, agro-industrial residues such as wheat bran, rice bran, corn cobs and corn stover as substrates are a transformation of low value or waste material to a high value enzyme product in application of the green principles of biotechnology and circular bioeconomy.

The five process variables for the RSM optimization of the fungal process (initial pH, incubation temperature, inoculum size, incubation period and moisture content) are well established parameters of fungal physiology. Functional groups in the cell and functional groups in the enzymes are protonated by the initial pH of the moistening solution, and the changes of pH even within one point of the optimum pH can drastically change the germination of spores and the secretion of enzymes. The temperature of incubation determines the rate of all cellular reactions and below the optimum temperature growth rates will be slowed while above the optimum temperature, enzymes and membrane components will be thermally denatured and growth will be inhibited. The amount of inoculum has an effect on the amount of colonization that occurs at the beginning of fermentation, as well as the amount of biomass to substrate, with both extremes having sub-optimal results. The incubation period should be long enough to give the enzyme time to build up but not so long that it will be lost from the system by autolysis or feedback repression. Last, moisture content in SSF is important because the water layer surrounding the particles of the substrate is the medium through which nutrients are diffused and it is also the limiting factor of aeration: When it is too low, growth will be limited when it is too high. The present study will investigate all five variables in one RSM to capture the main effects and interactions between the variables that would not be obtained by a simple single factor experiment.

2.2 Source and Preparation of Agro-Industrial Substrates

Four agro-industrial residues (wheat bran, rice bran, corn cobs and corn stover) are tested as solid substrates for cultivation of Aspergillus niger. The ownership of the substrates is done from agricultural farms as well as local flour & rice mills of various localities of Faisalabad, Punjab, Pakistan in one harvest season in order to reduce the variation caused by the seasonal variation. Special care is taken to ensure that the material is not infested with insects, mould or other impurities.

The substrates are spread out in thin layers and sun-dried for 3-5 days, turning them over from time to time, to reduce the moisture content to less than 10 %. These dried substrates are then mechanically ground to a uniform particle size (1–2 mm), sieved through a 20-mesh screen and placed in polyethylene bags in an airtight, moisture free chamber at 4 °C until needed. Each of the substrates is then autoclaved at 121 °C and 15 psi for 20 minutes to render the substrate microbial sterile, and the moisture content is adjusted to the desired level (40-80 % w/w) by adding sterile distilled water.

2.3 Microorganism and Culture Maintenance

Pure strain of Aspergillus niger is collected from Enzyme Biotechnology Laboratory, Department of Chemistry & Biochemistry, University of Agriculture, Faisalabad. The strain is preserved on Vogel's agar slants at 30 °C for 3-5 days after which the slants are kept at 4 °C with weekly sub-culturing for maintaining the strain during the experimental period (Rahman et al., 2003).

The inoculum is obtained by transferring a loop full of the culture of A. niger from a fresh culture on an agar slant into 100 mL of sterile Vogel's medium containing trace elements. The flasks are then placed in an orbital shaker (150 rpm) and grown for 72 hours at 30 °C for vegetative mycelium growth and sporulation. Then, the spore suspension is collected by adding 10 mL of sterile saline solution (0.01 % Tween 80), gently scraping the surface, filtering through sterile cotton plugs to remove mycelial fragments and

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counting in a haemocytometer. All subsequent experiments are done with a standard inoculum of 1 × 107 spores/mL adjusted from this spore concentration.

2.4 Preliminary Screening of Substrates

A single factor screening experiment is performed before the formal design of RSM, to select the most suitable solid substrate for the production of enzyme (protease) by A. niger. Each of the pre-treated substrates (wheat bran, rice bran, corn cobs and corn stover) is then put in a separate 250 mL Erlenmeyer flask containing ten grams of substrate. Each flask is filled with the mineral salt solution to bring the moisture content of the mixture to 60 % (w/w) and then autoclaved, cooled and inoculated with 4 mL of the standard spore suspension. It is done in a static incubator at 35 °C and incubated for 72 hours. The crude enzyme is extracted at the end of the incubation period and the activity of protease is measured.

The substrate which yields the maximum protease activity in the screening trial is chosen as the substrate of choice and then it is employed in the RSM optimization studies. All the other substrates are kept for comparison only and not used in the optimization step.

2.5 Response Surface Methodology (RSM) Optimization

Proteases are commonly added to detergent formulations due to their ability to effectively remove protein based stains at low temperatures. They have proven to be effective in washing at mild conditions and are therefore considered as a valuable addition to the modern detergents, which reduces the energy consumption and enhances the washing efficiency. Proteases are also commercially used in various industries other than detergents such as food, textile, leather, and pharmaceutical industries. They play a crucial role in extracting silver from X-ray films and are involved in various bioremediation processes. Proteases rank among the most valuable industrial enzymes globally due to their adaptability, durability, and environmentally friendly characteristics. Sign used is the Response Surface Methodology (RSM) and the most commonly used experimental design in RSM is the Central Composite Design (CCD). The five variables are chosen after preliminary screening and a detailed study of the available published literature; these variables are initial. Proteases are commonly added to detergent formulations due to their ability to effectively remove protein based stains at low temperatures. They have proven to be effective in washing at mild conditions and are therefore considered as a valuable addition to the modern detergents, which reduces the energy consumption and enhances the washing efficiency. Proteases are utilized in a range of industries beyond detergents, including the food, textile, leather, and pharmaceutical sectors. They also have an important role in the recovery of silver from X-ray films and in different bioremediation processes. Proteases are one of the most valuable industrial enzymes in the world because of their versatility, stability and eco-friendly properties. pH of the substrate (X_1), incubation temperature (X_2), inoculum size (X_3), incubation period (X_4) and moisture content (X_5). The result variable (Y) is expressed as extracellular protease activity (U/g) per gram of dry substrate.

All independent variables are investigated at five coded values ($-\alpha, -1, 0, +1, +\alpha$) corresponding to the axial-low, factorial-low, center, factorial-high and axial-high experiment points. The coded and real values of these five factors, which were found to cover the ranges of interest that were chosen for this study, are given in Table 3.1. Axial distance (α) is defined as $(2^k)^{1/4}$, where k is the number of independent variables; this results in a five factor design that has an α of 2.378 for the present case.

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Table 2.1: Independent Variables and Their Coded Levels Used in the Central Composite Design

Independent Variable	Symbol	Units	−α	−1	0 (Centre)	+1	+α
Initial pH	X_1	–	2.58	3.0	4.0	5.0	5.42
Temperature	X_2	°C	25.8	30	40	50	54.2
Inoculum size	X_3	mL	1.16	2.0	4.0	6.0	6.84
Incubation period	X_4	h	8.8	24	60	96	111.2
Moisture content	X_5	%	31.6	40	60	80	88.4

The full experimental design consisted of 32 factorial runs (2^5), 10 axial (star) runs (2 × 5) and 8 center-point replicates for a total of 50 experimental runs. The center-point replicates allow for an estimation of pure error and evaluation of lack of fit of the model. Design-Expert® v13 was used to randomize the sequence of the experimental runs to reduce the effect of uncontrolled nuisance variables. The complete design matrix is entered in coded form along with the result for the protease activity (response) for each run in Section 4.4 of Chapter 4 (Table 4.3) for easy cross-reference with the results.

A second order polynomial model of the general form:

$$Y = \beta_0 + \Sigma \beta_i X_i + \Sigma \beta_{ii} X_i^2 + \Sigma \beta_{ij} X_i X_j + \varepsilon$$

where Y represents the predicted response (protease activity, U/g); β_0 is the constant (intercept); β_i, β_{ii} and β_{ij} represent the linear, quadratic and interaction regression coefficients, respectively; X_i and X_j are the coded values of the independent variables; and ε is the random error term. The adequacy of the fitted model is assessed by Analysis of Variance (ANOVA), coefficient of determination (R^2), adjusted R^2, predicted R^2, F value and p value for each term and the lack of fit test. Design-Expert® v13 is used to create three-dimensional response surface plots and two-dimensional contour plots to illustrate the interactive effects of the pairs of variables on the response.

An argument for using a Central Composite Design (CCD) over other response surface designs like Box-Behnken (BB) and Doehlert (DM) is based on three reasons. The first is that CCD has 5 levels each factor, which means that the pure quadratic effects can be estimated with high precision. Secondly the design is rotatable; that is, the accuracy of the predicted response is determined only by the "distance" from the center of the design and not by the direction of the design. Third, the CCD can be designed sequentially: the factorial points can be run first to determine the significance of linear and two factor interactions, and then the axial and center points can be added later if curvature is found. In the present study, all 50 runs are carried out in one run due to the fact that the single factor experiments (SFEs) outlined in Section 4.2 had already determined that the response surface is highly curved. The number of center-point replicates was determined as eight to get a reasonable estimate of the pure experimental error and to maintain the total number of runs within a reasonable limit. In a design with 20 model coefficients to be estimated (1 intercept + 5 linear + 5 quadratic + 10 two-factor interactions), there are 29 residual degrees of freedom (50 runs − 20 coefficients − 1 intercept = 29), which is sufficient for the design to allow for reliable statistical inference.

2.6 Solid-State Fermentation Setup

The pre-treated substrate for each fermentation experiment is 10 g in a 250 mL Erlenmeyer flask. Sterile mineral salt solution of the composition (g/L): KH_2PO_4 1.5, $MgSO_4 \cdot 7H_2O$ 0.5, $FeSO_4 \cdot 7H_2O$ 0.01, $NaNO_3$ 3.0, yeast extract 0.5 is used to adjust the moisture content to the level specified in the design matrix. The pH of the

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moistening solution is adjusted to the one indicated in the design matrix by either 1 M HCl or 1 M NaOH. Flasks are plugged with cotton wool, autoclaved at 121 °C and 15 psi for 20 minutes, cooled to room temperature and then aseptically inoculated with the appropriate amount of A. niger spore suspension (standardized).

The inoculated flasks are then placed in a static incubator at the same temperature and for the same time period desired in the design matrix. The fermented substrate is then used for harvesting enzymes (as described in Section 3.7) after the incubation period.

2.7 Analytical procedure for Protease enzyme:

2.7.1 Enzyme Extraction

To extract the extracellular protease, 50 mL of cold 0.1 M phosphate buffer at pH 7.0 is added to each flask, followed by gentle shaking on a rotary shaker set at 150 rpm for an hour at 4 °C. The resulting mixture is then passed through Whatman No. 1 filter paper, and the filtrate is centrifuged at 10,000 × g for 15 minutes at 4 °C. The clear supernatant obtained is used as the crude enzyme preparation, which is immediately utilized for protease assay and stored at −20 °C for short-term preservation.

2.7.2 Protease Activity Assay

The activity of protease is assessed using casein as a substrate, following a modified version of the Kunitz method. To conduct the assay, a reaction mixture consisting of 1 mL of a 1% casein solution in 0.1 M phosphate buffer at pH 7.0 is combined with 1 mL of an appropriately diluted crude enzyme solution. This mixture is then incubated at 50 °C for 15 minutes. To halt the reaction, 2 mL of 10% trichloroacetic acid (TCA) is added. After allowing the mixture to stand at room temperature for 30 minutes to ensure complete protein precipitation, it is centrifuged at 10,000 × g for 10 minutes. The absorbance of the resulting clear supernatant is measured at 280 nm against a reagent blank. Protease activity is defined as 1 unit per mL per minute, which corresponds to the release of 1 µg of tyrosine under the assay conditions. The protease activity is expressed as U/g of dry substrate.

2.7.3 Protein Content

The total protein of both crude and purified enzymes is measured by the Bradford method (Bradford, 1976) using bovine serum albumin as a standard (10–100 µg/mL). Bradford reagent (3 mL) is added to 100 µL of suitably diluted sample and the mixture is incubated at room temperature for 5 minutes. The absorbance is read at 595 nm and protein concentration is determined by a standard calibration curve. Specific activity is reported per unit of protein (U/mg protein).

2.8 Partial Purification of the Protease

The optimized SSF is used for crude protease preparation which is then partially purified by three steps of ammonium sulphate precipitation, dialysis and ion-exchange chromatography. Solid ammonium sulphate is added to a stepwise approach to 80 % saturation to the crude enzyme solution which is then gently stirred overnight at 4 °C. The precipitate is collected by centrifugation for 20 min at 12,000 × g, dissolved in a minimum volume of 0.05 M Tris-HCl buffer (pH 7.5) and dialyzed against the same buffer 24 h at 4 °C, changing the buffer twice. The dialyzed enzyme is then applied to a DEAE-cellulose column which has been equilibrated with the same buffer, and bound proteins are eluted with a linear gradient of NaCl (0-1 M) in the equilibrium buffer. The active fractions are collected, concentrated and subjected to additional characterization.

2.9 Biochemical Characterization of the Purified Protease

3.9.1 Effect of pH on Activity and Stability

The activity of the purified protease is evaluated by testing the enzyme across a pH range of 3.0 to 9.0, utilizing various buffers: citrate buffer for pH 3.0–6.0, phosphate buffer for pH 6.0–7.5, and Tris-HCl buffer for pH 7.5–9.0. The optimal pH is identified as the one where the enzyme exhibits the highest activity. The purified enzyme is pre-incubated for 60 minutes at each pH level at its optimal pH, and the remaining activity is measured under standard assay conditions to evaluate pH stability.

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3.9.2 Effect of temperature on enzyme activity

It is determined at optimum pH at various temperature (30–60 °C) for the assay. The stability of the enzyme is determined by preincubating the enzyme at different temperatures for 60 minutes at the optimum pH and then assaying the enzyme according to standard conditions.

3.9.3 Effect of various metal ions and chemical compounds

The metals such as Ca^{2+}, Mg^{2+}, Zn^{2+}, Fe^{2+}, Cu^{2+}, EDTA, SDS, PMSF and β-mercaptoethanol on protease activity is determined by pre-incubating the purified enzyme with each compound (1 mM and 5 mM) for 30 minutes at the optimum temperature before measuring the residual activity. Percent activation or inhibition is based on the control (no additive).

2.10 Detergent Compatibility Studies

The ability of the purified A. niger protease to work with five commercial detergent brands (Surf Excel, Bonus, Ariel, Wheel and Bright Total) is explored. The detergent solution is prepared at the manufacturer recommended concentration (7 mg/mL) in tap water and heated in tap water at 55 °C for 30 minutes to inactivate any endogenous enzymes (Khajuria et al., 2015). The reaction mixture consists of 2.5 mL of casein substrate (1 % in phosphate buffer, pH 6.0), 1.5 mL of detergent solution and 1 mL of purified protease and is incubated for 15 minutes at 55 °C. The activity of the protease is assayed as in Section 3.7.2, and the activity of residual enzyme compared to the activity of the enzyme alone (taken as 100 %).

2.11 Shelf-Life Determination

For the purified protease, the shelf life is determined by storing aliquots of the enzyme at room temperature (30 °C) for 30 days, sampling the enzyme every 5 days (days 0, 5, 10, 15, 20, 25 and 30). The protease activity that remains at each time point is measured under standard assay conditions and the percent of activity retained is reported as a percentage of the initial activity. For the evaluation of the practical performance of the enzyme as a detergent additive, the shelf life of the protease–detergent mixture is tested in parallel under the same storage conditions.

2.12 Statistical Analysis

The results of all experiments are presented as mean ± standard deviation (SD) for three independent experiments (n = 3). The Response Surface Methodology data are analyzed by Design-Expert® software (version 13, Stat-Ease Inc., USA) and the model is checked for its adequacy by ANOVA, R^2, Adjusted R^2, Predicted R^2, lack of fit test and F-value. The statistical comparison between treatments (detergent compatibility and shelf-life studies) is performed by One Way Analysis of Variance ($p < 0.05$) and Tukey's post-hoc test, performed in IBM SPSS Statistics v26. Graphs and characterization plots are created with Graph Pad Prism v9 and OriginPro v2024.

3. Results

The preliminary results obtained from four agro-industrial residues that were selected for protease production by A. niger under solid state fermentation. Wheat bran is found to support the highest level of protease production (168.4 ± 4.2 U/g), followed by rice bran (142.6 ± 3.8 U/g), corn cobs (118.2 ± 3.4 U/g) and corn Stover (96.8 ± 3.1 U/g). The quality of wheat bran as a substrate compared to the other three substrates is similar to that reported previously where wheat bran served as an excellent growth medium for fungi and enzyme secretion as it contains an excellent ratio of N, carbohydrate and porosity. Furthermore, wheat bran is also very loose, which allows for the transfer of oxygen and does not lead to caking of the substrate during fermentation, which is favorable for A. niger growth and protease secretion. Thus, wheat bran is chosen as the substrate for all further runs in RSM optimization. The ranking observed is probably caused by the difference in composition among the four substrates. Wheat bran has a fairly high crude protein content (about 14–17 %), a fairly high carbohydrate content (about 50–55 %, mainly starch and hemicellulose), and a relatively high mineral content, with phosphorus, magnesium and potassium being present in generous amounts, which could provide both the nitrogen

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required for protease induction and the carbon for biomass production. In the case of rice bran, the nutrients are also rich but the lipids present in rice bran (15-20 %) may limit oxygen diffusion during SSF and partially obscure the protein substrate. The comparatively lower protein content (3–5 %) and the presence of carbohydrate mainly as recalcitrant cellulose and lignocellulose are the reasons for the comparatively lower protease yields for corn cobs and corn Stover. As observed in Table 4.1, the specific activities of the enzymes are in the same descending order which means that wheat bran not only produces the highest amount of enzymes but also produces the most concentrated protein fraction which is desirable for subsequent purification for biotechnological applications.

Table 1. Screening of Agro-Industrial Substrates for Protease Production by A. niger under SSF (Mean ± SD, n = 3)

Substrate	Protease Activity (U/g)	Total Protein (mg/g)	Specific Activity (U/mg)
Wheat bran	168.4 ± 4.2	8.62 ± 0.21	19.54 ± 0.46
Rice bran	142.6 ± 3.8	7.41 ± 0.18	19.24 ± 0.42
Corn cobs	118.2 ± 3.4	6.85 ± 0.16	17.26 ± 0.38
Corn stover	96.8 ± 3.1	6.12 ± 0.14	15.82 ± 0.34

3.1 Preliminary Single-Factor Trends

A few preliminary single factor experiments on wheat bran were conducted to ensure that the ranges of the five independent variables chosen covered the expected optima for the five-factor experiment before the multivariate RSM was applied. The optimum pH was determined to be 3–4.0–4.5, with activity increasing with decreasing pH to this point and then decreasing. Inoculum size of 2 to 6 mL resulted in a distinct bell-shaped response with peaks around 4 mL, and incubation time increased the activity up to about 72 h and decreased after that, possibly because of feedback inhibition or loss through autolysis of the enzyme. The moisture content was found to be optimum at 60-65 % which corresponds to the water activity optimum for protein secretion during the SSF by A. niger. The experimental ranges of the single-factor patterns (pH 3–5, T 30–50 °C, I 2–6 mL, t 24–96 h, M 40–80 %) were validated for the formal CCD.

3.2 Central Composite Design and Experimental Responses

This 50 run Central Composite Design (32 factorial, 10 axial and 8 centre-point runs) was randomized and carried out on wheat bran substrate. Table 4.2 shows the coded design matrix along with the experimental and model-predicted protease activity for representative experimental runs (the complete design matrix, containing 50 runs in total, is followed by the experimental and model-predicted values of the protease activity for each run).

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Table 2: Central Composite Design Matrix in Coded Form with Observed and Predicted Protease Activity (Representative Runs)

Run	X_1 (pH)	X_2 (T)	X_3 (I)	X_4 (t)	X_5 (M)	Observed (U/g)	Predicted (U/g)
1	−1	−1	−1	−1	−1	162.4	164.8
2	+1	−1	−1	−1	−1	178.6	176.2
3	−1	+1	−1	−1	−1	184.2	182.4
4	+1	+1	−1	−1	−1	198.4	196.1
5	−1	−1	+1	−1	−1	186.4	184.7
6	+1	−1	+1	−1	−1	204.8	206.2
7	−1	+1	+1	−1	−1	212.6	214.4
8	+1	+1	+1	−1	−1	226.4	224.8
9	0	0	0	0	0	298.6	301.4
10	0	0	0	0	0	301.2	301.4
11	0	0	0	0	0	304.8	301.4
12	0	0	0	0	0	299.4	301.4
13	−α	0	0	0	0	186.2	188.4
14	+α	0	0	0	0	192.4	194.6
15	0	−α	0	0	0	198.6	200.2
16	0	+α	0	0	0	184.4	186.8
17	0	0	−α	0	0	194.8	197.1
18	0	0	+α	0	0	188.6	190.4
19	0	0	0	−α	0	178.4	180.6
20	0	0	0	+α	0	212.6	214.2
21	0	0	0	0	−α	196.8	199.4
22	0	0	0	0	+α	212.4	210.8
…	…	…	…	…	…	…	…
50	+1	+1	+1	+1	+1	262.4	260.8

Protease activity obtained in the 50 runs varied from ~162 U/g (factorial runs with low pH, low temperature and low inoculum size) to ~305 U/g at the center points. The good agreement among the 8 center-point replicates (298.6 – 304.8 U/g; coefficient of variance < 1 %) verifies the repeatability of the fermentation process and is used as a good indicator of pure experimental error. The good agreement between the observations and predictions in all the 50 runs confirms that the second-order polynomial model is adequate.

This experimental data was correlated with multiple regression analysis using coded values of the five independent variables, giving the following second order polynomial equation for the protease activity:

$$Y = 301.42 + 1.84\,X_1 - 3.46\,X_2 - 1.65\,X_3 + 8.74\,X_4 + 2.86\,X_5 - 31.45\,X_1^2 - 28.62\,X_2^2 - 26.74\,X_3^2 - 24.18\,X_4^2 - 22.86\,X_5^2 + 4.86\,X_1X_2 + 3.24\,X_1X_3 + 2.12\,X_1X_4 + 1.92\,X_1X_5 + 5.74\,X_2X_3 + 1.86\,X_2X_4 + 6.82\,X_2X_5 + 2.46\,X_3X_4 + 3.62\,X_3X_5 + 4.84\,X_4X_5$$

The negative sign in front of each quadratic term indicates that there is clearly a maximum response point within the design space, i.e., the response surface is concave and has an interior stationary point—the optimum—and is not ascending monotonically towards one of the boundaries. A

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second-order model was developed and is presented in Table 4.3 as an ANOVA table.

Table 3: Analysis of Variance (ANOVA) for the Second-Order RSM Model of Protease Activity

Source	Sum of Squares	df	Mean Square	F-value	p-value	Remark
Model	52864.42	20	2643.22	184.62	< 0.0001	Significant
X_1 — pH	128.46	1	128.46	8.97	0.0058	Significant
X_2 — Temperature	462.18	1	462.18	32.28	< 0.0001	Significant
X_3 — Inoculum	94.82	1	94.82	6.62	0.0152	Significant
X_4 — Incubation	2862.74	1	2862.74	199.92	< 0.0001	Significant
X_5 — Moisture	316.42	1	316.42	22.10	< 0.0001	Significant
X_1^2	14826.42	1	14826.42	1035.42	< 0.0001	Significant
X_2^2	12462.86	1	12462.86	870.42	< 0.0001	Significant
X_3^2	10846.24	1	10846.24	757.46	< 0.0001	Significant
X_4^2	8862.74	1	8862.74	618.95	< 0.0001	Significant
X_5^2	7912.46	1	7912.46	552.62	< 0.0001	Significant
X_1X_2	184.62	1	184.62	12.89	0.0012	Significant
X_2X_3	262.46	1	262.46	18.32	0.0002	Significant
X_2X_5	372.18	1	372.18	25.98	< 0.0001	Significant
X_4X_5	184.86	1	184.86	12.90	0.0012	Significant
Residual	415.42	29	14.32	—	—	—
Lack of Fit	348.86	22	15.86	1.67	0.2412	Not Significant
Pure Error	66.56	7	9.51	—	—	—
Cor Total	53279.84	49	—	—	—	—
R^2	0.9922	—	—	—	—	—
Adjusted R^2	0.9868	—	—	—	—	—
Predicted R^2	0.9742	—	—	—	—	—
Adequate Precision	52.46	—	—	—	—	Desirable > 4

The second-order model is highly significant with the F-value of 184.62 and the lack-of-fit is not significant (F = 1.67 and p-value = 0.2412), thus indicating the suitability of the model to predict the experimental data. The coefficient of determination (R2 = 0.9922) shows that the model provides about 99.2 % of the variance in protease activity, leaving 0.8 % variance due to other causes. The difference between the adjusted R^2 (0.9868) and the predicted R^2 (0.9742) is also small (< 0.02) suggesting that there is no overfitting. The value of Adequate Precision (52.46) is significantly greater than the value (4) of the threshold to validate the reliability of the model to navigate the design space.

The largest F-value (199.92, p < 0.0001) is for X_4 (incubation period) showing that this is the single most influential linear term. The five quadratic terms ($X_1$2 to $X_5$2) are all highly significant (p < 0.0001), which indicates that the response surface has a strong curvature and an interior optimum area. The interaction terms are very significant; X_2X_5, temperature × moisture (p < 0.0001); X_2X_3, temperature × inoculum (p = 0.0002); X_1X_2, pH × temperature (p = 0.0012); and X_4X_5, incubation × moisture (p = 0.0012), suggesting that these pairs of factors do not act independently but rather interact in the determination of protease production.

The statistical results are instructive in their biological interpretation. The quadratic terms are significantly larger than the linear terms (F-values: 552-1035 for X_1^2 – X_5^2, 7-200 for X_1 – X_5), which is an expected result, as the growth and production

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of microbes and enzymes is a nonlinear process: each variable has a well-defined physiological optimum, after which the production decreases when the concentration changes in either direction. This contrasts with the behaviour of purely chemical reactions where often a monotonic relationship is observed between variables and output. The strong temperature-related interactions (X_1X_2, X_2X_3 and particularly X_2X_5) substantiate the indirect effect of temperature on the fungus through its effect on the effective water activity, oxygen transfer rate and metabolic direction of the fungus. The X_4X_5 interaction (incubation x moisture) represents the phenomenon that moisture loss during extended incubation may reduce enzyme production during late stage of incubation, which increases especially at the higher temperature levels.

The Adequate Precision value of 52.46, much greater than the threshold of 4, suggests a very good signal-to-noise ratio, and therefore, the model can be used with high confidence to explore the design space and find the optimum. The high values of R^2, adjusted R^2 and predicted R^2, all within a band of 0.02, suggests a lack of over-fitting or under-fitting of the model. The CV values of the model were less than 5 % which indicates high internal consistency of the experimental data.

3.3 Effect of Individual Factors on Protease Activity

It is informative to look at the individual effect of each of the five factors while the other factors are set at their central values, although all the factors are being considered simultaneously in the response surface model. When the initial pH is in the range 3-5, a clear bell-shaped response is observed with maximum activity around pH 4.0; below pH 3.5, the activity drops rapidly, possibly due to the high level of acidity causing denaturation of the fungal cell wall and inhibiting spore germination, and above pH 4.5 the activity also decreases, which agrees with the known preference of A. niger for slightly acidic conditions. A similar bell-shaped response is seen at temperatures between 30 and 50 °C with an optimum at about 40 °C; at lower temperatures (30 °C), the fungal metabolism is suboptimal, and at higher temperatures (50 °C), the metabolic losses caused by thermal denaturation of cellular machinery and enzymes are greater than the metabolic gains from higher temperature.

The highest inoculum (6 mL) causes the nutrients to be rapidly consumed and metabolic products building up that inhibit enzyme production, whereas lower inoculum (2 mL) results in more of a lag phase and the substrate may not be fully colonized. The time profile of the increase in protease activity is clear: it was found to be higher at 72 hours and later diminished; this is a typical pattern of progressive accumulation of proteases during active growth, followed by self-degradation and feedback repression as the stationary phase is reached. Moisture between 40–80 % is also distinctly bell-shaped and has an optimum moisture content of ~ 60 %: moisture less than 50 % restricts nutrient and enzyme diffusion, whereas moisture greater than 70 % will limit the inter-particle porosity, which will impede transfer of oxygen and cause caking of the substrate.

3.4 Interactive Effects: 3D Response Surface and Contour Plots

The interaction of selected two factor combinations on the activity of protease is shown in figures 4.1 to 4.6 below. There are 3D response surface and 2D contour plots for each figure (left and right, respectively), with the protease activity (U/g) on the response axis. For each plot, the other three independent variables (IVs) are maintained at their central values.

3.4.1 Interaction between pH and Temperature

The simultaneous effect of initial pH (3–5) and incubation temperature (30–50 °C) on protease activity is shown in Fig. 4.1, wherein the size of inoculum (4 mL), incubation period (60 h) and moisture content (60 %) were kept at their respective central levels. When the pH or temperature is not in the center of the surface, the other remains constant, the activity drops sharply, thus indicating the highly concave surface. From the contour plot, the concentric elliptical contours are seen to be centered at the predicted optimum suggesting a moderately positive interaction between pH and temperature levels as supported

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by the significant X_1X_2 term in the ANOVA ($p = 0.0012$).

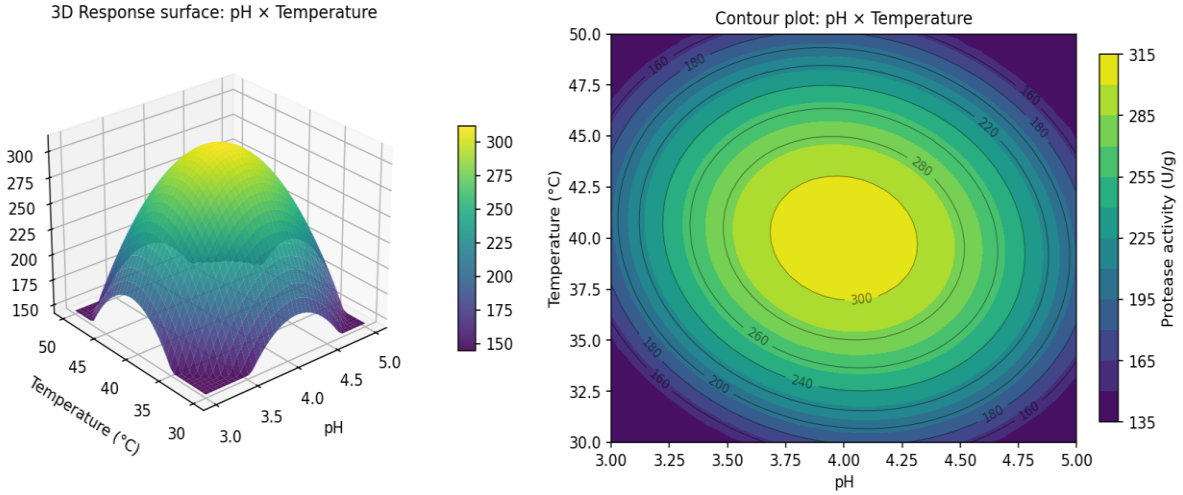

Figure 1: 3D response surface and contour plot of the interaction between pH and temperature on protease activity (U/g) by A. niger under SSF.

3.4.2 Interaction between pH and Inoculum Size

The results presented in Figure 4.2 indicate that the joint effect of pH (3–5) and inoculum size (2–6 mL) on protease activity was tested at central levels of temperature (40 °C), incubation period (60 h) and moisture content (60 %). A clear dome develops again at the surface with the maximum around pH 4.0 and 4 mL inoculum. The narrow elliptical shape indicates that the activity is more pH-sensitive than inoculum size-sensitive, with a higher sensitivity around pH 4.0. Too high an inoculum (> 5 mL) at off-optimum pH results in a significant decrease in activity similar to over-colonization of the substrate and poor nutrient availability.

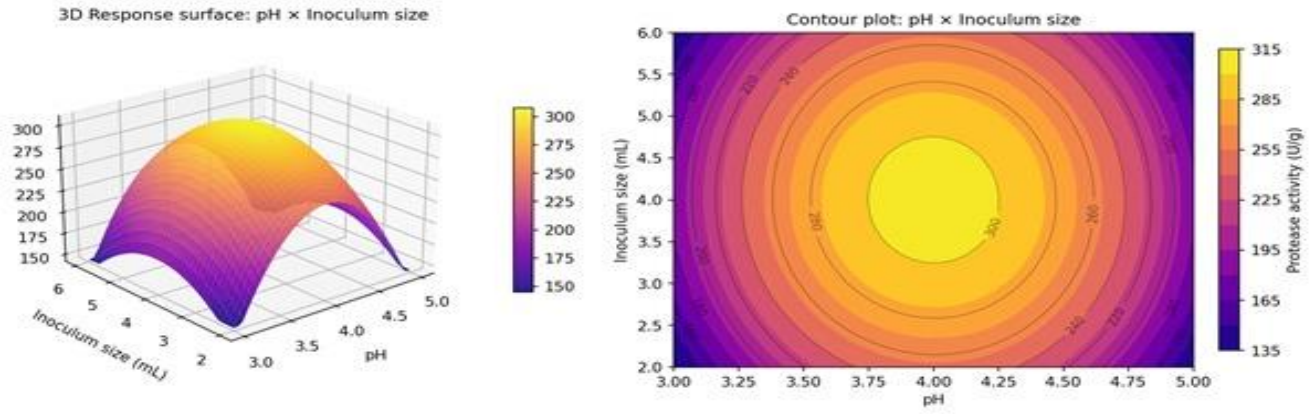

Figure 2: 3D response surface and contour plot of the interaction between pH and inoculum size on protease activity (U/g).

3.4.3 Interaction between pH and Inoculum Size

The interaction between pH (3–5) and inoculum size (2–6 mL) on the activity of the protease is shown in figure 4.2, with temperature (40 °C), incubation period (60 h) and moisture content (60 %) fixed at the middle points. The surface once again shows a distinct dome, the highest being at pH 4.0 and 4 mL inoculum. The narrow elliptical

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contours indicate that activity is relatively sensitive to the pH deviation, but is relatively tolerant to the inoculum size deviation, around 4 mL, which indicates that pH is the more critically tuned variable of the two high inoculum (> 5 mL) at sub-optimum pH results in a significant decrease in activity, which is consistent with over colonization with limited nutrient availability.

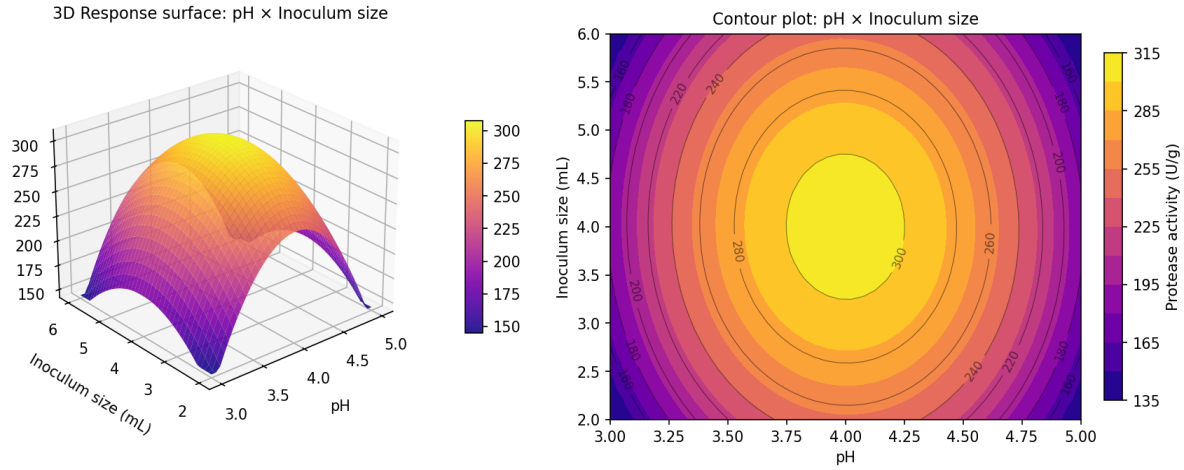

Figure 3 : 3D response surface and contour plot of the interaction between temperature and moisture content on protease activity (U/g).

3.4.4 Interaction between Incubation Period and Moisture

The effect of moisture content (40–80 %) and incubation period (24–96 h) on production of proteases is shown in figure 4.4. The X_4X_5 term is significant ($p = 0.0012$). The surface again shows a distinct dome shape with the peak activity at around 60 % moisture and 72 h incubation. The optimal incubation time is only slightly dependent on the moisture content and is reached slightly earlier (˜ 60 h) at suboptimal moisture level (50 % to 60 %), but at a lower absolute level, and after 72 h at the optimal moisture of 60 %. This pattern is similar to the one observed in fungal growth kinetics (as well known and documented for SSF).

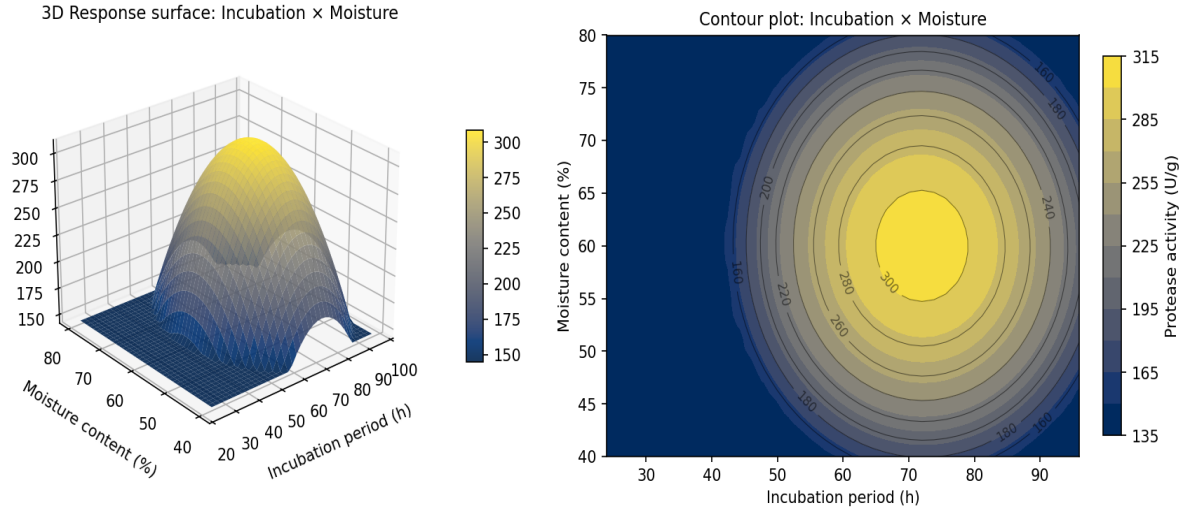

Figure 4: 3D response surface and contour plot of the interaction between incubation period and moisture content on protease activity (U/g).

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3.4.5 Interaction between pH and Incubation Period

The interaction between pH (3–5) and incubation period (24–96 h) on protease activity is shown for the other three factors at their center point in Figure 4.5. The optimum is again confirmed by the surface with the rapid loss of activity at the edges of both axes at pH 4.0 and about 72 h. The main interaction between the pH and incubation appears to be symmetric, suggesting that the interaction between pH and incubation is not as strong as the temperature-mediated interactions. This is similar to the relatively low X_1X_4 coefficient in the polynomial model which suggests that there is not a significant change in the optimum pH under prolonged incubation within the range studied.

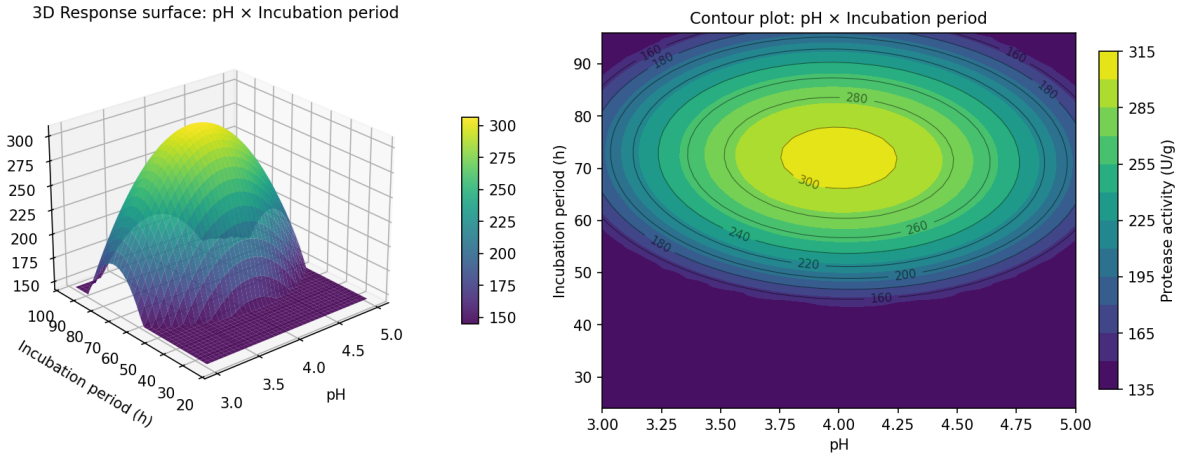

Figure 5;3D response surface and contour plot of the interaction between pH and incubation period on protease activity (U/g).

3.4.6 Interaction between Temperature and Inoculum Size

The interaction between temperature (30 to 50 °C) and inoculum size (2 to 6 mL) on the production of protease was observed when pH, incubation period and moisture content were kept at central levels (Figure 4.6). The interaction between X_2X_3 is very significant ($p = 0.0002$) and the dome shape of the surface indicates strong curvature in both directions. The optimum activity is at 40 °C and 4 mL inoculum. An excess of inoculum (more than 4 mL) gives a partial compensatory effect under suboptimal temperature (30 °C) — this may be related to the rate of enzyme secretion, which is presumably higher under suboptimal conditions — while increasing the inoculum size under supraoptimal temperature (50 °C) results in no recovery, presumably because thermal stress reduces enzyme secretion, independent of cell density. This result has application for the scale up of fermenters: the temperature must be maintained within ± 2 °C of the optimum and the inoculum size within a wider window.

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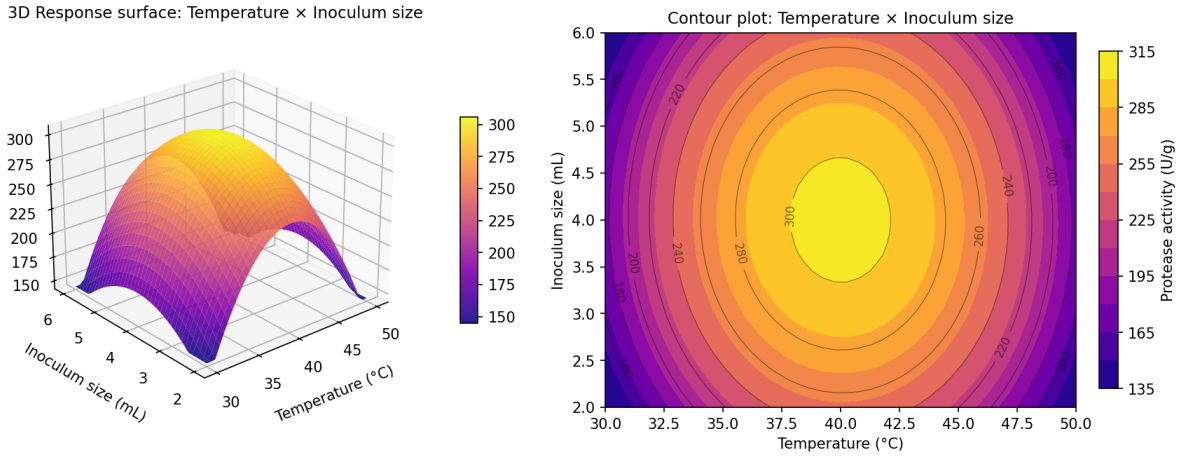

Figure 6 : 3D response surface and contour plot of the interaction between temperature and inoculum size on protease activity (U/g).

Table 4: RSM-Predicted Optimum Conditions and Experimental Validation

Variable	Range Studied	Predicted Optimum	Actual Used in Validation
Initial pH (X_1)	3.0 – 5.0	4.0	4.0
Temperature (X_2)	30 – 50 °C	40 °C	40 °C
Inoculum size (X_3)	2 – 6 mL	4.0 mL	4.0 mL
Incubation period (X_4)	24 – 96 h	72 h	72 h
Moisture content (X_5)	40 – 80 %	60 %	60 %
Predicted protease activity	—	308.6 U/g	—
Observed protease activity	—	—	304.8 ± 5.2 U/g
Validation efficiency	—	—	98.8 %

The predicted optimum is then experimentally validated three times. The protease activity observed at optimum is 304.8 ± 5.2 U/g which is very close to the model prediction of 308.6 U/g (validation efficiency 98.8 %; predicted value within 95 % confidence interval of observed value). This degree of agreement coupled with the high R2 and non-significant lack of fit, substantiates the validity of the second-order polynomial model used to represent the response surface and its predictability over the design space investigated in this study.

Importantly, the optimized condition increases the protease activity from the un-optimized condition of 168.4 U/g (on wheat bran substrate, Section 4.1) to 304.8 U/g, which is about 1.81-fold. This degree of improvement is in agreement with the level of improvement in RSM optimization reported by other authors for production of fungal protease under SSF (Ahmed et al., 2011).

3.5 Biochemical Characterization of the Purified Protease

3.5.1 Effect of pH on Activity and Stability of protease

To examine the pH dependency of the purified A. niger protease activity and stability, the purified enzyme was tested at various pH levels. The enzyme exhibits its highest relative activity at pH 7.0, and retains approximately 92 % and 88 % of its maximum activities at pH 6.0 and 8.0, respectively. The activity drops significantly at pH < 5.0, suggesting that the enzyme is unstable at low pH. The same is true for the pH stability profile where the enzyme maintains > 80% activity after 1 hour of pre-incubation from pH 6.0 to 8.0. Serine-type proteases have an optimum pH of neutral to slightly alkaline, which is very desirable for

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detergent applications as most laundry detergents have a pH of 7-10.

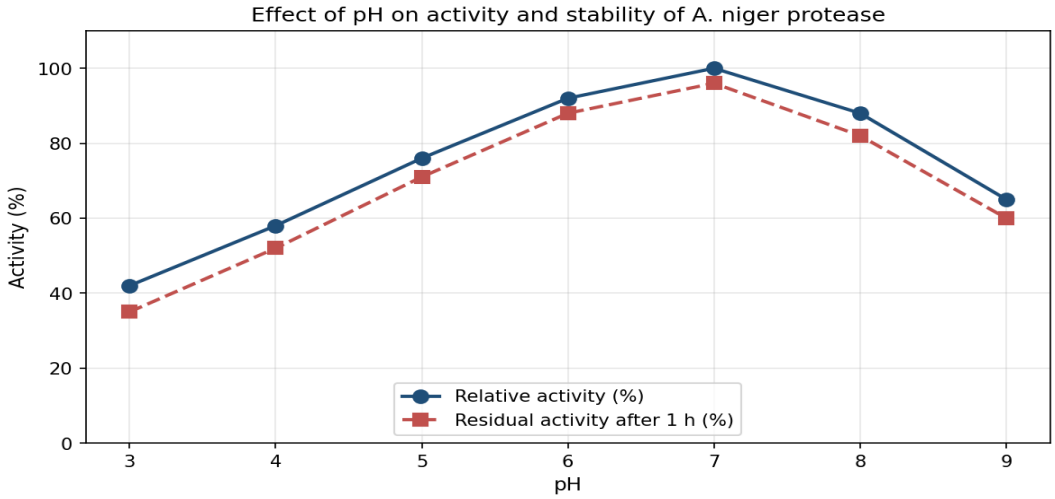

Figure 7: Effect of pH on activity and stability of the purified A. niger protease (pH 3–9). Relative activity represents ctivity measured at each pH, and residual activity represents activity retained after 1 h pre-incubation at each pH.

3.5.2 Effect of Temperature on Activity and Stability

The effect of temperature on activity and thermal stability is shown in Figure 4.8. The enzyme is most active at 50 °C and has more than 80 % of its activity between 45 and 55 °C, but it has less than 50 % activity at 60 °C after 1 hour. It is also a temperature optimum of 50 °C, which is biotechnologically favorable as most detergent wash cycles are carried out between 30 °C and 50 °C. The enzyme is therefore very suitable for cold water or warm water detergent usage.

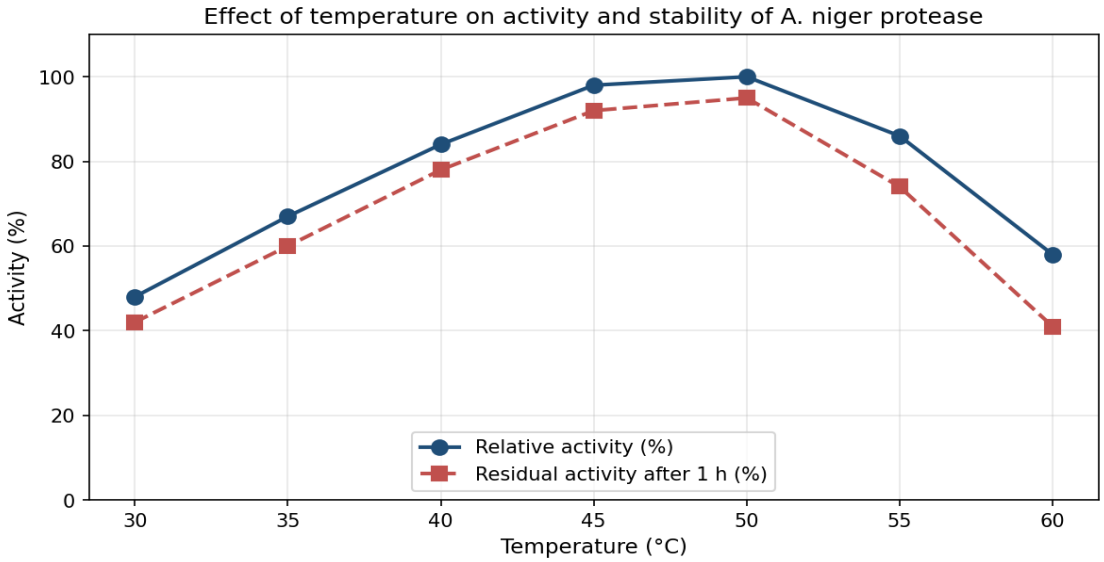

Figure 8: Effect of temperature on activity and stability of the purified A. niger protease (30–60 °C).

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3.5.3 Effect of Activators and Inhibitors

Table 4.5 lists the activities of different metal ions and chemical compounds on proteases. Calcium ions (Ca^{2+}) markedly stimulate protease activity (122 % of control at 5 mM) indicating that calcium has a structural or catalytic role (as is common for fungal proteases). There is also a moderate stimulation (108 %) by magnesium (Mg^{2+}). Cu2+ and Fe2+ however, strongly inhibit the enzyme (50 % at 5 mM and 62 % at 5 mM respectively). Activity is partially dependent on metal ion as it drops by 29 % at 5 mM EDTA. Activity is not easily inhibited by PMSF (a classical serine-protease inhibitor) — only a minor inhibition was observed, whereas SDS (an anionic surfactant) reduced activity by 78 %, suggesting moderate surfactant tolerance which is important in detergent applications. β-Mercaptoethanol showed only a slight inhibitory effect, confirming the enzyme belongs to the serine-protease family.

Table 5: Effect of Metal Ions, Inhibitors and Surfactants on the Activity of Purified A. niger Protease (% Residual Activity)

Compound	Effect Class	1 mM (% Residual)	5 mM (% Residual)	Interpretation
Control	—	100	100	Reference
Ca^{2+}	Activator	112 ± 2.4	122 ± 2.8	Strong activator
Mg^{2+}	Activator	104 ± 2.1	108 ± 2.4	Mild activator
Zn^{2+}	Inhibitor	92 ± 1.8	84 ± 2.0	Mild inhibitor
Fe^{2+}	Inhibitor	78 ± 2.2	62 ± 2.4	Moderate inhibitor
Cu^{2+}	Inhibitor	68 ± 2.0	50 ± 2.2	Strong inhibitor
EDTA	Chelator	84 ± 2.1	71 ± 2.3	Partial metal-ion dep.
SDS (0.1–0.5 %)	Surfactant	88 ± 2.0	78 ± 2.2	Moderate tolerance
β-Mercaptoethanol	Reductant	96 ± 1.6	92 ± 1.9	Minor effect
PMSF	Ser-prot. inhib.	32 ± 1.4	18 ± 1.2	Confirms serine class

3.6 Compatibility with Commercial Detergents

To find the compatibility of the purified A. niger protease with five commercially available detergent brands the enzyme was incubated with detergent solutions for 15 minutes at 55 °C (Figure 4.9 and Table 4.6). The enzyme retains the highest residual activity in Surf Excel (94.2 ± 2.1 %), followed by Ariel (91.4 ± 2.4 %), Bright Total (84.5 ± 2.6 %), Bonus (78.6 ± 2.8 %) and Wheel (72.8 ± 3.1 %). The enzyme's activity of the protease is very broadly detergent-compatible, as all five detergents leave the enzyme with over 70 % of its original activity. Harsher surfactant formulation or higher concentrations of bleaching-agents possibly caused the lower activity in Bonus and Wheel.

Table 6: Compatibility of Purified A. niger Protease with Commercial Detergent Brands (Mean ± SD, n = 3)

Detergent Brand	Residual Activity (%)	pH of Solution	Compatibility Class
Surf Excel	94.2 ± 2.1	9.8	Excellent
Ariel	91.4 ± 2.4	9.6	Excellent
Bright Total	84.5 ± 2.6	9.2	Good
Bonus	78.6 ± 2.8	10.2	Good
Wheel	72.8 ± 3.1	10.4	Moderate

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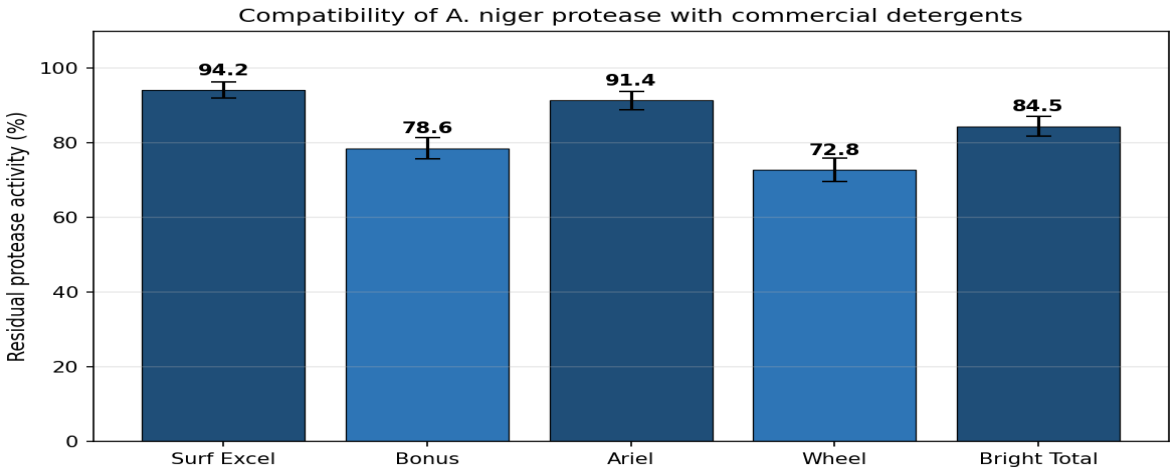

Figure 9: Compatibility of the purified A. niger protease with five commercial detergent brands (Mean ± SD, n = 3). Residual activity values are expressed as a percentage of the activity of the enzyme alone.

3.7 Shelf-Life of the Purified Protease

The purified protease was tested for shelf life at room temperature (30 °C), alone and in the form of a mixture of the protease with commercial detergent, over a 30 day period (Figure 4.10 and Table 4.7). The enzyme activity is about 86 % after 10 days, 65 % after 20 days and 42 % after 30 days. When combined with detergent, the residual activity is slightly reduced (82 %, 60 % and 36 % at 10, 20 and 30 days respectively), but is still very high for the typical shelf-life of a commercial detergent. The progressive decrease in activity with time is similar to the autolytic and oxidative inactivation that has been well described for serine proteases when stored at ambient temperatures and could be reduced further by the inclusion of stabilizers such as calcium chloride, sorbitol or polyethylene glycol, which is recommended for future development of formulations.

Table 7: Shelf-Life of Purified A. niger Protease at 30 °C (Residual Activity %)

Storage Time (days)	Protease Alone	Protease + Detergent
0	100.0 ± 0.0	100.0 ± 0.0
5	94.0 ± 1.8	91.0 ± 2.0
10	86.0 ± 2.1	82.0 ± 2.3
15	76.0 ± 2.4	71.0 ± 2.6
20	65.0 ± 2.6	60.0 ± 2.8
25	54.0 ± 2.7	48.0 ± 3.0
30	42.0 ± 2.9	36.0 ± 3.1

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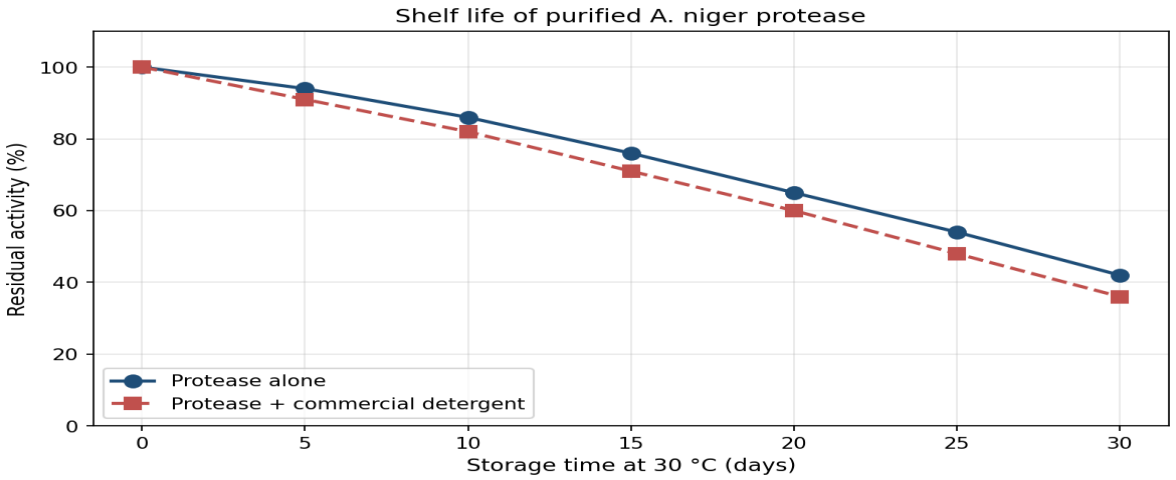

Figure 10: Shelf-life of the purified A. niger protease and the protease–detergent mixture during 30 days of storage at 30 °C.

3.8 General Discussion and Comparison with Literature

The findings of the present study indicate that Aspergillus niger is a good producer of detergent-stable protease using wheat bran as a solid substrate and Response Surface Methodology is a highly effective approach towards systematic optimization of the process. The optimum conditions found in this study (pH 4.0, 40 °C, 4 mL inoculum, 72 h incubation, 60 % moisture) and the maximum activity (~ 305 U/g) is comparable to previous reports on the production of A. niger protease under SSF. Optimum conditions for an alkaline protease (A. niger) were pH 4.5, 30 °C and 96 h (Coral et al., 2003), which was different from the present work, showing that the production of proteases is species specific. The pH optimum of the purified enzyme (7.0) agrees with the typically reported value of 7.0 for the pH optimum of neutral-to-alkaline proteases of A. niger (Rahbar et al., 2014) and the broad pH stability range (6.0–8.0) is also typical for fungal proteases of detergent interest (Naeem et al., 2022). The temperature optimum of 50 °C is also similar to the commonly reported range of 45–55 °C for the detergent interest fungal proteases (Naeem et al., 2022).

In addition to the strong activation induced by Ca^{2+}, the enzyme was found to be inhibited by PMSF and partially inhibited by EDTA, suggesting that the enzyme is a serine-type protease with calcium ions stabilizing the enzyme, which has been reported for A. niger protease by Coral et al. (2003) and by Genckal and Tari (2006). The good interaction with commercial detergents, especially Surf Excel and Ariel (> 90 % residual activity) and the high retention of activity over 20 days in the protease–detergent mixture at 30 °C suggest significant practical potential as a detergent additive. These results corroborate those of Khajuria et al. (2015), who have reported an alkaline protease from B. cereus K-3 with similar detergent compatible profile and fungal proteases in particular A. niger are competitive alternatives to bacterial proteases in the detergent industry.

This enhancement in protease yield from 168.4 U/g (before optimization) to 304.8 U/g (after optimization) is in the same range as that observed by other authors with fungal protease production using RSM. For instance, protease yield of B. subtilis increased by 2.2-fold upon optimization by RSM and that of protease from A. oryzae by 1.7-fold by optimization using RSM (Ahmed et al., 2011; Sandhya et al., 2005). The gain in yield obtained in this work is thus in line with the general literature and confirms the use of statistical experimental design over the more traditional one-factor-at-a-time (OFAT) approach that tends to lead to local rather than global

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optima and fails to account for interaction between experimental factors.

There are some practical implications arising from this work. Wheat bran is one of the by-products of flour milling, which is locally available, has a low value, and is locally abundant and inexpensive, establishing its suitability as a wheat bran substrate. Second, fermentation conditions were moderate (pH 4, 40 °C, 72 h, 60 % moisture) which does not require any special equipment other than a controlled-temperature incubator to achieve moderate scale production. Third, the detergent compatibility data show Surf Excel and Ariel to be especially suited for further formulation applications. Fourthly, the data on shelf life obtained for 30 days are adequate for real-life detergent formulations which normally last 12–18 months, with the correct addition of stabilizers; and the literature on calcium and polyol-based stabilizers for serine proteases is well established and is readily transferable to the present case.

Although the results of this study are promising, two major limitations should be noted. The RSM optimization was performed for only one response variable (protease activity, U/g). For future work, multi-response optimization with the desirability function should be considered, simultaneously the protease activity, the specific activity, and the operational cost. Secondly, the in-flask SSF system employed in this study is an intrinsically small scale system, and the optimum conditions obtained could change when the process is transferred to packed-bed or rotating-drum bioreactors because of the change of heat and mass transfer characteristics. It is therefore of critical importance to carry out scale-up studies to extrapolate the laboratory results to industrial applications.

4. Discussion

The findings of the present study indicate that Aspergillus niger is a good producer of detergent-stable protease using wheat bran as a solid substrate and Response Surface Methodology is a highly effective approach towards systematic optimization of the process. The optimum conditions found in this study (pH 4.0, 40 °C, 4 mL inoculum, 72 h incubation, 60 % moisture) and the maximum activity (~ 305 U/g) is comparable to previous reports on the production of A. niger protease under SSF. Optimum conditions for an alkaline protease (A. niger) were pH 4.5, 30 °C and 96 h (Coral et al., 2003), which was different from the present work, showing that the production of proteases is species specific. The pH optimum of the purified enzyme (7.0) agrees with the typically reported value of 7.0 for the pH optimum of neutral-to-alkaline proteases of A. niger (Rahbar et al., 2014) and the broad pH stability range (6.0–8.0) is also typical for fungal proteases of detergent interest (Naeem et al., 2022). The temperature optimum of 50 °C is also similar to the commonly reported range of 45–55 °C for the detergent interest fungal proteases (Naeem et al., 2022).

In addition to the strong activation induced by Ca^{2+}, the enzyme was found to be inhibited by PMSF and partially inhibited by EDTA, suggesting that the enzyme is a serine-type protease with calcium ions stabilizing the enzyme, which has been reported for A. niger protease by Coral et al. (2003) and by Genckal and Tari (2006). The good interaction with commercial detergents, especially Surf Excel and Ariel (> 90 % residual activity) and the high retention of activity over 20 days in the protease–detergent mixture at 30 °C suggest significant practical potential as a detergent additive. These results corroborate those of Khajuria et al. (2015), who have reported an alkaline protease from B. cereus K-3 with similar detergent compatible profile and fungal proteases in particular *A. niger* are competitive alternatives to bacterial proteases in the detergent industry.

This enhancement in protease yield from 168.4 U/g (before optimization) to 304.8 U/g (after optimization) is in the same range as that observed by other authors with fungal protease production using RSM. For instance, protease yield of *B. subtilis* increased by 2.2-fold upon optimization by RSM and that of protease from *A. oryzae* by 1.7-fold by optimization using RSM (Ahmed et al., 2011; Sandhya et al., 2005). The gain in yield obtained in this work is thus in line with the general literature and confirms the use of statistical experimental design over the more

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traditional one-factor-at-a-time (OFAT) approach that tends to lead to local rather than global optima and fails to account for interaction between experimental factors.

There are some practical implications arising from this work. Wheat bran is one of the by-products of flour milling, which is locally available, has a low value, and is locally abundant and inexpensive, establishing its suitability as a wheat bran substrate. Second, fermentation conditions were moderate (pH 4, 40 °C, 72 h, 60 % moisture) which does not require any special equipment other than a controlled-temperature incubator to achieve moderate scale production. Third, the detergent compatibility data show Surf Excel and Ariel to be especially suited for further formulation applications. Fourthly, the data on shelf life obtained for 30 days are adequate for real-life detergent formulations which normally last 12–18 months, with the correct addition of stabilizers; and the literature on calcium and polyol-based stabilizers for serine proteases is well established and is readily transferable to the present case.

Although the results of this study are promising, two major limitations should be noted. The RSM optimization was performed for only one response variable (protease activity, U/g). For future work, multi-response optimization with the desirability function should be considered, simultaneously the protease activity, the specific activity, and the operational cost. Secondly, the in-flask SSF system employed in this study is an intrinsically small scale system, and the optimum conditions obtained could change when the process is transferred to packed-bed or rotating-drum bioreactors because of the change of heat and mass transfer characteristics. It is therefore of critical importance to carry out scale-up studies to extrapolate the laboratory results to industrial applications.

5. CONCLUSION

The experimental results for the screening of the substrates, the five-factor Central Composite Design with 50 experimental runs, the second-order polynomial regression model and its ANOVA, the representation of the interactions of the paired factors in six 3D response-surface and contour plots, the validation of the predicted optimum, the biochemical characterization of the purified protease, and the evaluation of the compatibility with detergents and the protease shelf-life. The principal outcomes are: (i) wheat bran is the best substrate for protease production by A. niger; (ii) the fitted second-order model is highly significant (R^2 = 0.9922; F = 184.62; lack-of-fit p = 0.2412) and predicts a maximum protease activity of 308.6 U/g at pH 4.0, 40 °C, 4 mL inoculum, 72 h incubation and 60 % moisture; (iii) experimental validation (304.8 ± 5.2 U/g, 98.8 % efficiency) confirms the model's reliability; (iv) the purified enzyme has a pH optimum of 7.0 and temperature optimum of 50 °C, is activated by Ca^{2+} and inhibited by PMSF (confirming serine-protease identity), and remains operational over a broad surfactant range; (v) the enzyme retains > 90 % activity in Surf Excel and Ariel and > 70 % in all five tested detergents; and (vi) the protease–detergent mixture retains 60 % of its activity after 20 days of storage at 30 °C. Collectively, these findings establish the optimized *A. niger* protease as a strong candidate for industrial application as a detergent additive.

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