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ORIGINAL PAPER

Acid protease production by solid-state fermentation using *Aspergillus oryzae* MTCC 5341: optimization of process parameters

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Abstract *Aspergillus oryzae* MTCC 5341, when grown on wheat bran as substrate, produces several extracellular acid proteases. Production of the major acid protease (constituting 34% of the total) by solid-state fermentation is optimized. Optimum operating conditions obtained are determined as pH 5, temperature of incubation of 30°C, defatted soy flour addition of 4%, and fermentation time of 120 h, resulting in acid protease production of 8.64×10^5 U/g bran. Response-surface methodology is used to generate a predictive model of the combined effects of independent variables such as, pH, temperature, defatted soy flour addition, and fermentation time. The statistical design indicates that all four independent variables have significant effects on acid protease production. Optimum factor levels are pH 5.4, incubation temperature of 31°C, 4.4% defatted soy flour addition, and fermentation time of 123 h to yield a maximum activity of 8.93×10^5 U/g bran. Evaluation experiments, carried out to verify the predictions, reveal that *A. oryzae* produces 8.47×10^5 U/g bran, which corresponds to 94.8% of the predicted value. This is the highest acid protease activity reported so far, wherein the fungus produces four times higher activity than previously reported [J Bacteriol 130(1): 48–56, 1977].

Keywords Acid protease · Solid-state fermentation · *Aspergillus oryzae* · Media optimization

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Introduction

Proteases occupy a central position in commerce, accounting for nearly 65% of the global enzyme market. They are used extensively in the detergent, leather, pharmaceutical, and food industries [21]. Food applications of proteases include their use in cheese-making, beer clarification, protein hydrolysate production, pharmaceutical, and cosmetic industries [3]. Acid proteases find application in the production of seasoning materials, protein hydrolysates, fermentation of soy sauce, and as digestive aids [21].

Filamentous fungi are exploited for the production of industrial enzymes due to their ability to grow on solid substrate and produce a wide range of extracellular enzymes. Among the many advantages offered by the production of enzymes by fungi are low material costs coupled with high productivity, faster production, and the ease with which the enzymes can be modified. Further, the enzymes, being normally extracellular, are easily recoverable from the media. Although several reports have appeared recently about isolation of acid proteases from different fungi, *Aspergillus oryzae* is an organism of choice, due to its generally regarded as safe (GRAS) status [11]. There is ample scope for searching for new molecular species of acid proteases, since many reported activity levels are poor [6, 23, 26, 31].

In the conventional method, media is optimized by changing one variable at a time while keeping other factors at a constant level [22], which is laborious and often leads to wrong conclusions. Multivariate experiments are designed not only to reduce the number of experiments necessary in the optimization process but also to produce more defined results than those available by univariate strategies [8]. Response-surface methodology (RSM) is one such multivariate analysis tool comprising mathematical

and statistical techniques for generating empirical models. It evaluates the effects of the individual factors and provides optimal levels of variables for desirable responses. RSM is customarily used as a statistical tool in the majority of media optimization studies [5].

The objective of this work is to optimize factors influencing acid protease production by using RSM. As a part of purification and characterization of proteases from *A. oryzae* MTCC 5341, sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) and zymogram confirm the release of one dominant acid protease [32]. The acid protease from *A. oryzae* MTCC 5341 has been purified with a yield of 29%, having a specific activity of 43,658 U/mg [32]. The enzyme prefers hydrophobic amino acids at S_1 and S_1' positions and also activates trypsinogen to trypsin [32].

Optimization of process parameters for solid-state fermentation (SSF) is carried out to target this acid protease. In a conventional one-parameter-at-a-time fashion we identify four parameters (pH, temperature, fermentation time, and defatted soy flour addition) affecting the yield of the desired enzyme.

Materials and methods

The fungus used in this present study (*A. oryzae* MTCC 5341), isolated at the Central Food Technological Research Institute (CFTRI) and deposited in the Microbial Type Culture Collection, IMTECH, Chandigarh, was periodically subcultured and maintained on potato dextrose agar slant. Fungal spores were dispensed into sterile saline solution to prepare the inoculum for fermentation. This spore suspension was serially diluted to obtain a spore density of 10^5 spores/mL. One milliliter of this suspension was used to inoculate 100 g dry substrate.

Hemoglobin (denatured) was from MP Biomedical Inc. (Solon, OH); trichloroacetic acid (TCA) was from Himedia Laboratories Pvt. Ltd. (Mumbai, India). All other chemicals used were of analytical grade.

Substrate preparation and fermentation

Cereal brans, wheat, rice, and ragi (*Eleucine coracana*) were purchased from local market of Mysore, India, and were of commercial grade. Initial moisture content of the bran was estimated by an infrared moisture meter wherein the bran was dried to a constant weight at 105°C (Sartorius AG, Flawil, Switzerland). Solid-state fermentation (SSF) was carried out in 500-mL Erlenmeyer flasks. A mineral solution containing 70 mg each of $CuSO_4{\cdot}5H_2O$, $ZnSO_4{\cdot}7H_2O$, and $FeSO_4{\cdot}7H_2O$ in 100 mL 0.2 N HCl was added to the bran to obtain pH 5.0 (4 mL/30 g bran). The final moisture content of the bran was adjusted to 60% with mineral solution before autoclaving. Erlenmeyer flasks containing 30 g substrate were autoclaved at 121°C for 40 min and inoculated with 1 mL spore suspension containing 10^5 spores/mL. The flasks were incubated at 28–30°C and 90–95% relative humidity (RH). The activity of the acid protease was checked at intervals of 24 h during 192 h of fermentation. The moldy bran was dried at 50°C in a tray drier to a moisture content of less than 6%, and stored at 4°C. The bran was extracted with 0.1 M NaCl in water [ratio of bran-to-solvent, 1:10 (w/v)], when required. The solution was filtered through a sterile filter membrane (0.22 μm) to obtain an enzyme solution free of any solid material, free of culture at 4°C.

Screening of the factors affecting protease production

Effect of carbon sources

Bran from cereals, such as wheat, rice, and ragi (Finger millet), were used as complex sources of carbon and nitrogen for the fungus to produce acid protease. The brans were autoclaved at 121°C for 40 min before adding fungal spore suspension solution. The pH of the bran was 5.0. Inoculated flasks were incubated at 30°C. The extent of enzyme production was monitored as a function of time.

Effect of nitrogen sources

To further increase the protein content in the solid substrate (bran), complex sources of nitrogen such as defatted soy flour, defatted sesame flour, casein, and peptone were added to the bran. The effect of individual source on protease production was tested at five different levels; 2%, 4%, 6%, 8%, and 10% (w/w).

Effect of chemical additives on acid protease production

In order to study the effect of different additives on protease production, wheat bran was supplemented with sterile phytic acid (0.4%), Al_2Cl_3 (0.1 mM), and biotin (0.1%) in water (passed through 0.22-μm filters). The inoculum was added and flasks were incubated at 30°C for 120 h. At the end of the period, the extent of enzyme production was determined.

Effect of cultivation time, incubation pH, and temperature on acid protease production

Wheat bran was moistened to 60%, autoclaved, and inoculated with fungal spores as described above. The extent of enzyme production was determined every 24 h for a total period of 192 h.

The effect of pH on acid protease production by SSF was carried out at pH 3, 4, and 5. Media pH was adjusted using dilute HCl. The activity of the enzyme was monitored every 48 h.

Temperature for growth and production of acid protease was optimized by incubating the flasks containing the inoculated bran at 25°C, 30°C, 35°C, and 40°C. The acid protease activity was monitored every 48 h.

Optimization of factors affecting protease production by response-surface methodology

Experimental design

The critical parameters that could affect the production of acid protease were determined by initial experiments to be: pH, incubation temperature, defatted soy flour addition, and fermentation time. Response-surface methodology (RSM) was used for studying effects of interaction among these variables. The optimized ranges for the selected variables were pH 3–7, incubation temperature of 25–35°C, addition of defatted soy flour of 0–8%, and fermentation time of 72–168 h. Box-Behnken design [9] was adopted to optimize the levels of the four factors, with three center points yielding a set of 27 experiments. The factors at three different levels (−1, 0, +1) with minimum and maximum range of values were as presented in Table 1. The treatment schedule for the model is given in Table 2. Three replicates (treatments 13–15 in Table 2) at the center of the design were used for estimation of the pure error sum of squares. The experiments were randomized to maximize the effects of unknown variability due to irrelevant factors in the observed responses.

Statistical analysis

Average maximum acid protease activity was taken as the dependent variable (response), with duplicates. The average maximum acid protease activity was taken as the dependent variable. The response value (Y) in each trial was the average of duplicates. A second-order polynomial equation, fitted to the data by multiple regression procedure, resulted in an empirical model. For the four-factor system, the following model equation was used:

$$Y = \beta_0 + \beta_1 A + \beta_2 B + \beta_3 C + \beta_4 D + \beta_{11} A^2 + \beta_{22} B^2 + \beta_{33} C^2 + \beta_{44} D^2 + \beta_{12} AB + \beta_{13} AC + \beta_{14} AD + \beta_{23} BC + \beta_{24} BD + \beta_{34} CD + \varepsilon, \quad (1)$$

where Y is the predicted response for acid protease produced; β_0 is the value of the fitted response at the center point of the design; β_1, β_2, β_3, and β_4 are the linear coefficients; β_{11}, β_{22}, β_{33}, and β_{44} are the quadratic coefficients; β_{12}, β_{13}, β_{14}, β_{23}, β_{24}, and β_{34} are the interaction coefficients; while ε is the random error. The software package Design-Expert® 7.0 (Stat Ease, Inc., Minneapolis, USA), was used to obtain the coefficients of Eq. 1 based on the data provided in Table 2. The responses under different combinations as defined by the design (Table 2) were analyzed using analysis of variance (ANOVA) to estimate the statistical parameters.

Table 1 Experimental range and levels of the four independent variables used in RSM in terms of actual and coded factors

Variables	Levels		
	−1	0	+1
pH	3	5	7
Temperature (°C)	25	30	35
Defatted soy flour addition (%)	0	4	8
Fermentation time (h)	72	120	168

Protease assay

Acid protease activity was determined using 2% hemoglobin (acid denatured) in glycine–HCl (pH 3.2, 0.1 M) as substrate, as reported earlier [24]. In short, activity was determined by incubating 1 mL substrate with 400 µL of appropriately diluted enzyme solution for 10 min at 55°C. Enzyme activity was arrested by the addition of 2 mL of 5% TCA. The unhydrolyzed protein was removed by filtration (Whatman no. 1). Absorbance of the supernatant was measured at 280 nm. One unit was defined to be the amount of enzyme that produced an increase in absorbance of 0.001/min under the above conditions. Protein concentration was determined by Lowry's method [17] using bovine serum albumin as standard.

Results and discussion

A. oryzae MTCC 5341 produces an acid protease that has been purified to homogeneity with molecular weight of 47 kDa [32]. The enzyme shows endoproteolytic activity and prefers L–V, Y–T, and I–K residues at the S_1 and S_1' positions. To optimize the conditions for production of this enzyme, choice of cereal bran, fermentation time, pH of substrate, temperature of incubation, source and amount of protein supplementation, and inclusion of chemical additives in substrate were studied. These are described below.

Table 2 Experimental design used in the RSM studies of four independent variables with three centre points for acid protease production by *A. oryzae* MTCC 5341 by SSF

Run order	A pH	B Temperature (°C)	C Defatted soy flour concentration (%)	D Fermentation time (h)	Mean observed response ($\times 10^5$ U/g bran)
1	+1	0	0	+1	3.6
2	0	0	−1	+1	3.2
3	−1	0	−1	0	1.42
4	+1	0	−1	0	2.35
5	0	+1	0	−1	4.21
6	0	0	+1	+1	4.23
7	+1	−1	0	0	2.82
8	0	0	−1	−1	2.01
9	0	−1	0	+1	1.64
10	+1	0	+1	0	5.21
11	0	+1	0	+1	5.28
12	0	−1	+1	0	2.21
13[a]	0	0	0	0	8.64
14[a]	0	0	0	0	8.64
15[a]	0	0	0	0	8.66
16	−1	−1	0	0	0.43
17	+1	0	0	−1	3.71
18	0	+1	−1	0	4.67
19	0	0	+1	−1	2.96
20	−1	+1	0	0	2.0
21	0	−1	0	−1	1.79
22	−1	0	0	−1	1.01
23	−1	0	0	+1	1.41
24	0	+1	+1	0	4.09
25	+1	+1	0	0	4.9
26	−1	0	+1	0	2.31
27	0	−1	−1	0	1.72

[a] Central value

Selection of cereal bran for SSF

Wheat bran, rice bran, and ragi (*E. coracana*) bran were used as media for solid-state fermentation of *A. oryzae* MTCC 5341. Wheat bran was the best medium, with acid protease activity of 4.8×10^5 U/g dry bran (100%). When grown on rice bran and ragi bran, the fungus produced 3.57×10^5 U/g (74.4%) and 0.5×10^5 U/g bran (11.6%), respectively (Fig. 1a). Since wheat bran has higher protein content (14–16%) compared with rice bran (7.5–8%) and ragi bran (6–7%), the production of protease was probably enhanced due to the higher protein content in the substrate. Accordingly, wheat bran was preferred as the substrate for SSF.

Effect of source and amount of additional protein on protease production

The choice of bran revealed that acid protease production was enhanced with higher protein content in the media. Protein sources such as defatted soy flour (45–50% protein); defatted sesame flour (50–55% protein), casein, and peptone were supplemented to the wheat bran to study their induction of enzyme activity. The protein sources were individually tested at five different levels: 2%, 4%, 6%, 8%, and 10% (w/w). A positive response was seen at all the levels tested. Soy flour at 4% was found to increase the activity level from 4.8×10^5 U/g bran to 8.26×10^5 U/g bran. Sesame flour (7.65×10^5 U/g bran), casein (6.0×10^5 U/g bran), and peptone (5.70×10^5 U/g bran) also increase protease production, albeit to a lesser extent (Fig. 1b). Higher levels of protein (> 4%) sources repressed the activity.

Effect of additives on protease production

SSF in presence of additives such as biotin (0.1%), phytic acid (0.4%), and aluminium chloride (0.1 mM) reportedly enhances protease production [30]. However, in the present study, biotin, phytic acid, and aluminium chloride did not significantly influence acid protease production (Fig. 1b).

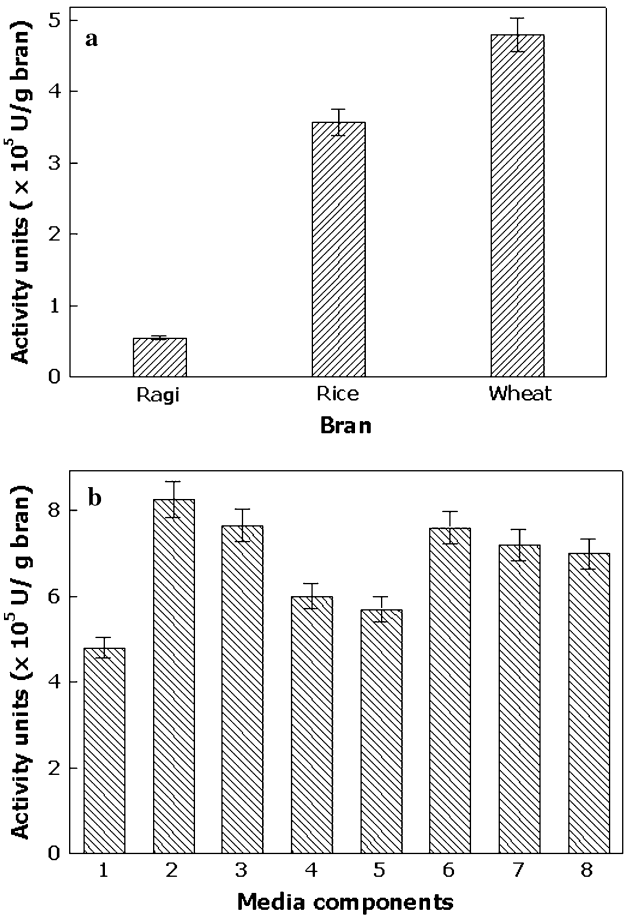

Fig. 1 **a** Screening of cereal brans for suitability as substrate in SSF for acid protease production by *A. oryzae* MTCC 5341. The fungus was cultivated on different cereal brans for 120 h. At the end of fermentation, acid protease activity was determined. **b** Optimization of media components for growth and production of extracellular acid proteases. Treatments include: wheat bran (WB) alone as media incubated at pH 5 (1), wheat bran + 4% defatted soy flour (DSF) (2), wheat bran + 4% defatted sesame flour (3), wheat bran + 4% casein (4), wheat bran + 4% peptone (5), WB + 4% DSF + 0.1% biotin (6), WB + 4% DSF + 0.4% phytic acid (7), and WB + 4% DSF + 0.1 mM Al_2Cl_3 (8). Fermentation was done at 30°C and pH 5.0 for a period of 120 h. Each value is the average of three experiments

Effect of cultivation time, incubation pH, and temperature on acid protease production by *A. oryzae*

Acid protease production was monitored as a function of time of cultivation on wheat bran with addition of 4% defatted soy flour (50% protein). Enzyme production gradually increased with fermentation time. At the end of 120 h, 8.26×10^5 U/g bran was obtained. Fermentation after 120 h resulted in slight reduction in the activity level, reaching a value of 7.45×10^5 U/g bran at 144 h. Fermentation time was optimized as 120 h (Fig. 2a).

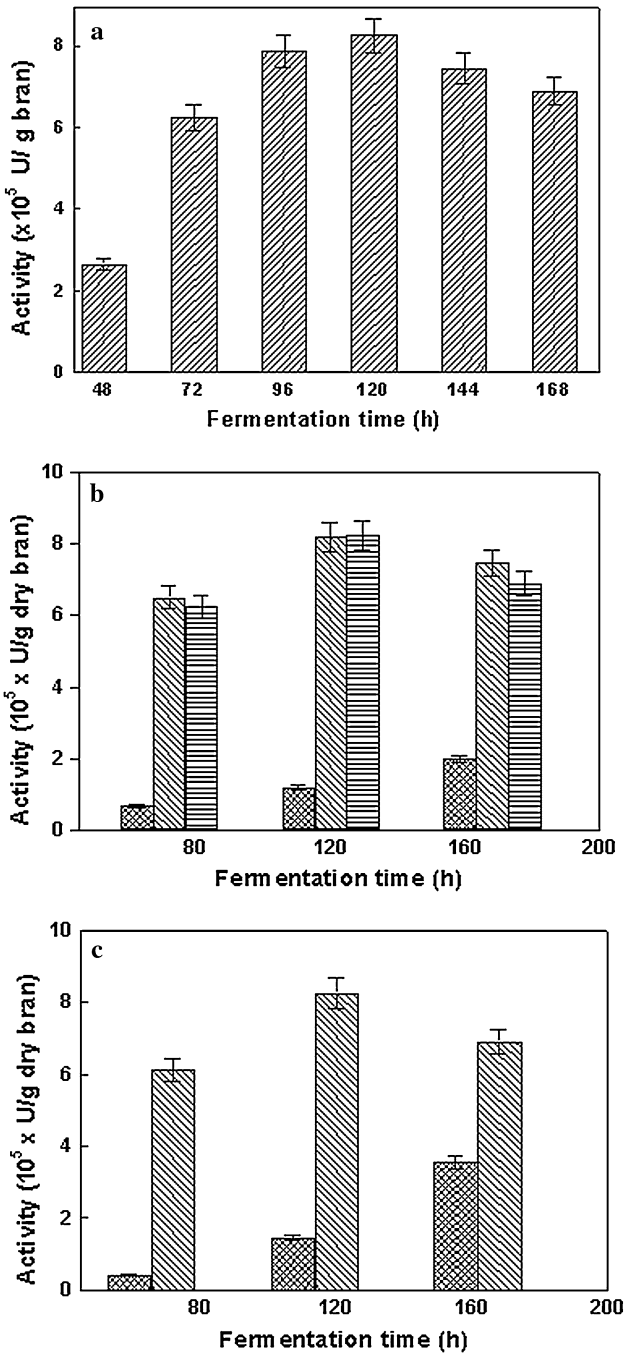

Fig. 2 **a** Production of acid protease by *A. oryzae* as a function of time. At the end of each fermentation time, moldy bran was extracted with water containing 0.1 M NaCl for 60 min. Activity was determined as described in the "Protease assay" section. **b** Effect of pH on growth and production of acid protease from *Aspergillus oryzae* MTCC 5341. pH of solid media during growth was 3 (▨), 4 (▨), and 5 (▤). **c** Effect of temperature on growth and production of acid protease from *Aspergillus oryzae* MTCC 5341. Temperature of incubation during growth was 25°C (▨) and 30°C (▨)

The optimum pH of medium for production of extracellular protease activity was 4–5, resulting in an activity of 8.26×10^5 U/g bran (Fig. 2b). Growth at pH 3 was

Table 3 Analysis of variance for the fitted second-order polynomial model and lack of fit for acid protease production as per Box-Behnken design

Source	Sum of squares	*df*	Mean sum of squares	*F*-value	Prob > *F*	Significance
Model	130.1695	14	9.297	29.911	<0.0001	Significant
pH (*A*)	16.356	1	16.356	52.62	<0.0001	
Temperature (*B*)	17.617	1	17.617	56.677	<0.0001	
Defatted soy flour (*C*)	2.6508	1	2.6508	8.527	0.0128	
Fermentation time (*D*)	1.122	1	1.122	3.610	0.0817	
AB	0.065	1	0.065	0.290	0.655	
AC	0.9702	1	0.9702	3.121	0.102	
AD	0.065	1	0.065	0.2091	0.655	
BC	0.2862	1	0.2862	0.9208	0.356	
BD	0.3721	1	0.3721	1.197	0.295	
CD	0.0016	1	0.0016	0.00514	0.944	
A^2	58.417	1	58.417	187.935	<0.0001	
B^2	39.918	1	39.918	128.422	<0.0001	
C^2	37.689	1	37.689	121.249	<0.0001	
D^2	42.550	1	42.550	136.889	<0.0001	
Residual	3.730	12	0.3108			
Lack of fit	3.729	10	0.3729	2797.356	<0.0001	Not significant
Pure error	0.00026	2	0.00013			
Corr. total	133.899	26				

slow, with low enzyme production (3.2×10^5 U/g bran at the end of 192 h).

Temperature of incubation during growth had a significant influence on protease production. Temperature of 25°C was suboptimal for growth, whereas growth ceased above 35°C. The optimum temperature range for growth and protease production was determined to be 30°C (Fig. 2c). It was also observed that, when bran was incubated at 25°C, enzyme production yield gradually increased up to the end of 160 h (3.57×10^5 U/g bran).

Based on the above results, it was concluded that pH of the substrate, fermentation time, temperature of incubation, and amount of defatted soy flour supplemented in the substrate were the critical factors for acid protease activity. The effect of these factors and their interaction for production of acid protease were optimized by response-surface methodology.

Optimization of factors influencing protease production by RSM using Box-Behnken design

Interactive effects of the factors, viz., pH, incubation temperature, defatted soy flour addition, and fermentation time, were examined by RSM using Box-Behnken design. The actual yield of acid protease (response) obtained is presented in Table 2. The ANOVA analysis yielded the following regression equation in terms of the levels of acid protease produced (Y) as a function of pH (A), temperature of incubation (B), defatted soy flour addition (C), and fermentation time (D).

$$\begin{aligned}\text{Acid protease activity } (\times 10^5 \text{ U/g bran}) &= 8.65 + 1.17 \times A + 1.21 \times B + 0.47 \\ &\times C + 0.31 \times D + 0.13 \times A \times B + 0.49 \times A \\ &\times C - 0.13 \times A \times D - 0.27 \times B \times C + 0.31 \\ &\times B \times D + 0.02 \times C \times D - 3.31 \times A^2 - 2.74 \\ &\times B^2 - 2.66 \times C^2 - 2.82 \times D^2.\end{aligned}$$

The subsequent analysis of variance showed aptness of the model for acid protease production. The computed F-value of 29.911 implies significance of the model. There is only a 0.01% chance that a model F-value this large could occur due to noise. The lack-of-fit F-value is not significant, and there is only a 37.29% chance that a lack-of-fit F-value this large could occur due to noise. The model was found to be highly significant and sufficient to represent the actual relationship between the response and the significant variables as indicated by the small model P-value (<0.0001), large lack-of-fit P-value (0.3729), suitable coefficient of determination ($R^2 = 0.9721$), and adjusted coefficient of determination ($R^2_{\text{adjusted}} = 0.9396$) from ANOVA (Table 3). The predicted sum of squares (PRESS) of 21.48 indicated fit of each point in this design. Significance of seven model terms (A, B, C, A^2, B^2, C^2, and D^2)

Table 4 Analysis of variance (ANOVA) table for response-surface quadratic model

Parameter	Value
Standard deviation	0.56
Mean	3.52
R^2	0.9721
Adjusted R^2	0.9396
Predicted R^2	0.8395
F-value	29.91
PRESS	21.48
Adequate precision	19.966

and an adequate precision of 19.996 indicated low signal-to-noise ratio (a ratio greater than 4 is desirable) (Table 4).

Response-surface curves for the variation in yield of acid protease as a function of two variables, with the other two kept at their central value, were constructed. The three-dimensional response surfaces obtained were convex in nature, suggesting well-defined optimum operating conditions. The sharp convexity implied that the responses vary significantly from the single-variable optimized conditions. From the response for the interaction of pH with temperature of incubation (Fig. 3a), protease yields increased with increasing pH and temperature up to a value of 5.0 and 30°C, respectively. Decreased enzyme activity was observed at pH above 5.0 and temperatures above 30°C. At optimum levels of these variables (pH 4–5 and 30°C), a maximum enzyme yield of 8.64×10^5 U/g bran was obtained. Defatted soy flour (50% protein) addition had a favorable impact on enzyme yield up to 4% (Fig. 3b). Accordingly, 4% protein and pH 4–5 were optimal for maximum protease production. Any further increase in its concentration repressed enzyme yield. Similarly the yields of acid protease were determined to be maximum at fermentation time of 120 h, beyond which activity declined (Fig. 3c). A convex-shaped response-surface curve was obtained when defatted soy flour addition was combined with temperature, indicating positive influence of these variables on acid protease production (Fig. 3d).

Analysis of response-surface curves and contour plots indicated optimum levels of the variables necessary to achieve better results. The results predicted by Box-Behnken design showed that a combination of pH 5, temperature of 30°C, and 4% defatted soy flour would favor maximum protease production, yielding 8.64×10^5 U/g bran after 120 h of fermentation. Thus, by optimizing the fermentation parameters using RSM, the yield of acid protease increased from 4.8×10^5 U/g bran to 8.64×10^5 U/g bran. Available reports indicated that the optimum pH and temperatures for growth fall in the ranges of 3–4 and 30–32°C, beyond which protease production drastically reduces [12, 28].

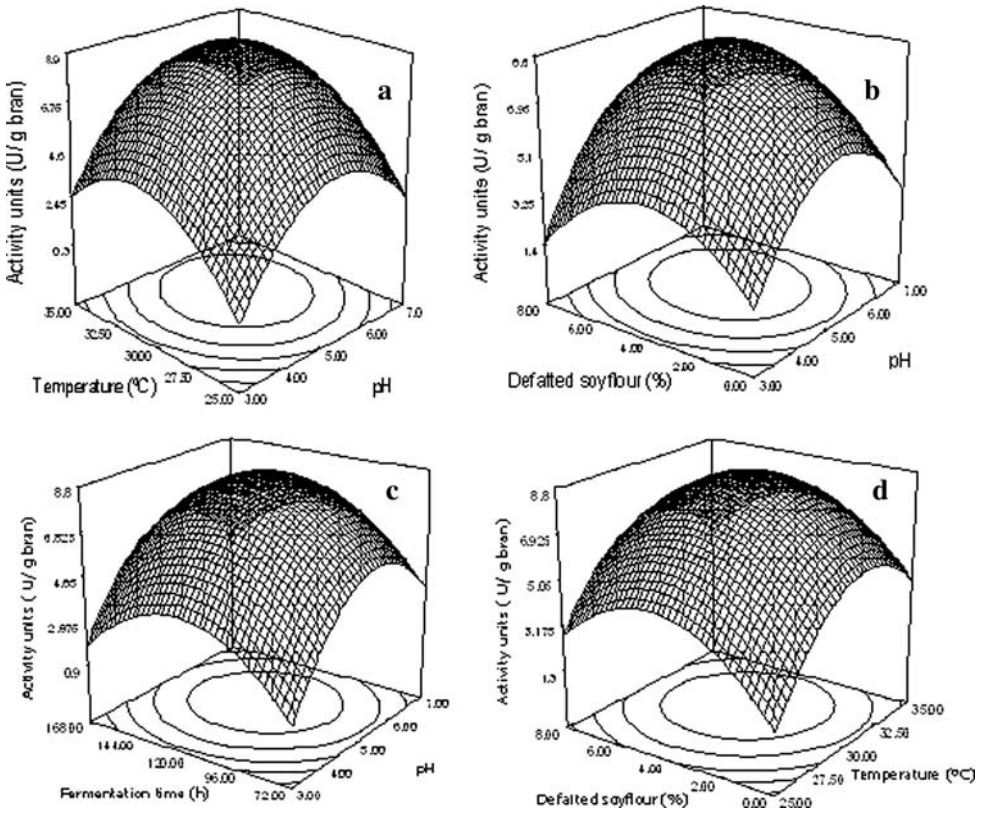

Fig. 3 **a** Response-surface curve of acid protease production from *Aspergillus oryzae* MTCC 5341, showing the interaction of temperature and pH after 120 h of incubation. Defatted soy flour supplementation and fermentation time were kept at their central points. **b** Acid protease production ($\times 10^5$ U/g bran) observed as a response to the interaction of defatted soy flour addition (%) and pH as variables. Temperature and fermentation time are kept at their central points. **c** Response-surface plot of acid protease production ($\times 10^5$ U/g bran) by *Aspergillus oryzae* MTCC 5341 showing interaction of fermentation time (h) and pH at central values of temperature and addition of defatted soy flour. **d** Acid protease production ($\times 10^5$ U/g bran) observed as a response to the interaction of defatted soy flour addition (%) and temperature as variables. Fermentation time and pH are kept at their central points

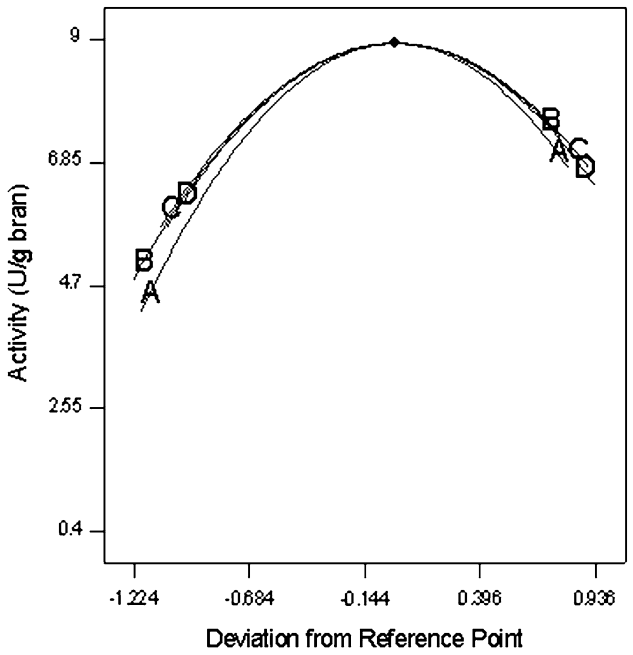

Fig. 4 Desirability graph for testing the efficacy of the model for production of acid protease ($\times 10^5$ U/g bran) from *Aspergillus oryzae* MTCC 5341. Factors included in the study are A = pH of incubation, B = temperature (°C), C = defatted soy flour addition (%), and D = fermentation time (h)

In an attempt to test the desirability of the model, the maximum value of the factors were determined as pH 5.4, incubation temperature of 31°C, defatted soy flour addition of 4.4%, and fermentation time of 123 h, yielding a maximum activity of 8.93×10^5 U/g bran (Fig. 4). Thus the central values framed were close to the values predicted by the statistical model.

In a separate set of evaluation experiments carried out to verify the predictions, *A. oryzae* produced 8.47×10^5 U/g bran, which is 94.8% of the predicted value. Table 5 shows predicted and observed responses to random levels of variables, wherein the two responses are similar in most cases.

A comparison of the specific activities of acid proteases from fungi is given in Table 6. Acid protease from the current study reports four times higher specific activity than the earlier reports from the same source [27] (Table 6).

It is interesting to note that, although a number of substrates have been tried in solid-state fermentation, wheat bran has been the preferred choice. When wheat bran is used solely for the production of proteases, it has resulted in low enzyme activities [10, 14, 15, 20]. In a report of acid protease production by *A. niger* Tieghem 331221, addition of protein sources such as casitone, casein, peptone, and trader's protein increased activity levels of the enzyme, whereas various carbon sources inhibited protease biosynthesis, thereby indicating the presence of catabolic repression of protease biosynthesis [2]. In yet another study, utilizing cheap protein sources for protease production, *Mirabilis jalapa* seed powder was used for increasing the alkaline protease activity yield (*Aspergillus clavatus* ES1) by a 14-fold increment (56 U/ml) over unoptimized media [19]. In a study on alkaline protease production by *Shewanella oneidensis* MR-1, optimization of media parameters by RSM was carried out by adding glucose (12.5 g L^{-1}) and tryptone (12.5 g L^{-1}) to the solid media, which increased protease production by 60% (112.9 U/mL) [1]. To the best of our knowledge, there are no reports of optimization of media components for acid protease production from *A. oryzae* by solid-state fermentation. Although RSM is used as a statistical tool for optimizing media components for protease production by other microorganisms such as *Neosartorya fischeri* [33], *Microbacterium* sp. [25] *Bacillus licheniformis* ATCC 21415 [18], and *Bacillus*

Table 5 Design for validation of predictive model using random levels of experimental factors

Run order	pH	Temperature (°C)	Defatted soy flour addition (%)	Fermentation time (h)	Observed response ($\times 10^5$ U/g bran)	Predicted response ($\times 10^5$ U/g bran)
1	4	25	1	100	0.95	0.977
2	6.5	30	6	80	5.17	5.27
3	5	30	4	120	8.47	8.647
4	3	35	4	96	1.13	1.16
5	5	28	4	80	5.51	5.609
6	5.5	32	6	120	8.32	8.36
7	6	30	5	140	8.02	8.02
8	7	28	5	72	2.74	2.72
9	5	30	4	120	8.45	8.64
10	4	30	4	120	8.20	8.05

Table 6 Comparison of specific activity of acid protease from *A. oryzae* MTCC 5341 with other reports

No.	Source	Substrate	Specific activity (U/mg)	Unit definition	Reference
1	*A. oryzae*	Trypsinogen	0.106	1 U = 1 μmol trypsinogen/min	[7]
2	*A. oryzae*	Casein	4.1	1 U = 1 μmol tyrosine/min	[28]
3	*A. oryzae*	Casein	1.6	1 U = Unit increase in $A_{280\ nm}$/30 min	[13]
4	*A. oryzae*	Casein	2.6	1 U = 0.1 increase in $A_{650\ nm}$/10 min	[16]
5	*A. clavatus*	Hemoglobin	2.2	1 U = 0.1 increase in $A_{280\ nm}$/60 min	[26]
6	*A. kawachi*	Casein	1.2	1*katal* U = 1 μg tyrosine/sec	[34]
7	*A. oryzae*	Casein	5.4	1 U = 1 μmol tyrosine/min	[29]
8	*A. oryzae*	Casein	43.1	1 U = 1 μmol tyrosine/min	[27]
9	*A. niger*	Hemoglobin	3,500	1 HUT = 1.1 μg tyrosine/min	Amanopro
	A. niger		1,242		
10	Pepsin	Hemoglobin	4,000	1 U = 0.001 increase in $A_{280\ nm}$/min	[24]
	Mucor renninus		5,500		
11	*A. oryzae* MTCC 5341	Hemoglobin	21,175	1 U = 0.001 increase in $A_{280\ nm}$/min	Current study
			172.04	1 U = 1 μmol tyrosine/min	
			8,928.27	1HUT = 1.1 μg tyrosine/min	

spp. [4], *A. oryzae* has not been reported using this model. Response-surface methodology offered stable responses in predicting the combined interactions of the four variables (pH, temperature, defatted soy flour addition, and fermentation time) with respect to extracellular acid protease production.

Conclusions

We report a study involving *A. oryzae* MTCC 5341 for the production of extracellular acid protease by solid-state fermentation. Response-surface methodology was adopted to optimize the critical variables and to study their influence on protease production. Response-surface curves indicate that optimum factor levels were pH 5.4, incubation temperature of 31°C, defatted soy flour addition of 4.4%, and fermentation time of 123 h, which would yield a maximum activity of 8.93×10^5 U/g bran. Evaluation experiments carried out to verify the predictions revealed that *A. oryzae* produces 8.47×10^5 U/g bran, which is 94.8% of the predicted value. This paper proposes a low-cost medium formulation that could be of industrial value, since acid proteases find potential applications in pharmaceutical and food industries.

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