



Improvement of protease production by *Rhizopus oryzae* CH4 grown on wheat gluten using response surface methodology and Its Scale-up in a Bioreactor

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ABSTRACT

In this work, three factors, five-level central composite design was used to optimize protease production by *Rhizopus oryzae* CH4 grown on wheat gluten. Gluten concentration, starch concentration and inoculums sizes significantly affected protease production. The optimal combinations of media constituents for maximum protease production (266.5 UP ml⁻¹) were determined as gluten concentration 22.5 g l⁻¹, starch concentration 30 g l⁻¹ and inoculums size 5x10⁶ spores ml⁻¹. Under the proposed optimized conditions, the protease experimental yield (266.5 UP ml⁻¹) closely matched the yield predicted by the statistical model (254.4 UP ml⁻¹) with R²=0.981. An overall 2.9-fold increase in enzyme production was achieved in the optimized medium. On subsequent scale-up in a 20-l bioreactor using conditions optimized through RSM, 258.2 UP ml⁻¹ of protease was produced in 60-72 h. This clearly indicated that the model remained valid even on a large scale. The relatively higher protease production by *Rhizopus oryzae* CH4 showed promise of offering great potential as additives in the bread making industry.

Key words: Media optimization; protease; response surface methodology; gluten; *Rhizopus oryzae*.

INTRODUCTION

Microbial proteases play an important role in biotechnological processes and they account for approximately 59% of the total enzymes used [1]. Proteases are produced by a wide range of microorganisms including bacteria, moulds and yeasts. Fungal protease is one of the major reasons for the wide popularity of fungi in fermentation industry. They find application in modern and biochemical industries; food, environmental and pharmaceutical processing.

Nowadays, Proteolysis of food proteins by microbial enzymes has been suggested to reduce their allergenic potential and produce hypoallergenic products [2-5]. During microbial fermentations proteolytic enzymes can be produced and they can degrade milk protein allergens [4]. Recent studies [6, 7] showed that pools of lactic acid bacteria supplemented with fungal proteases under specific processing conditions (long-time and semi-liquid fermentation) had the capacity to hydrolyze the wheat gluten improving their digestibility (Disease known as gluten intolerance or celiac disease). Fungal proteases routinely used in bakery industry, are indispensable to start the primary proteolysis of gluten [7]. Among fungi, proteases of *Aspergillus oryzae* and *A. niger*, used for bakery applications have been found to degrade gluten polypeptides [7]. The baked goods manufactured by the mixture of lactobacilli and fungal protease were not toxic for celiac disease patients [8].

Stepaniak et al. [9] proposed protease from *Aspergillus niger* as an oral supplement to reduce the gluten intake in celiac disease patients. The same approach was investigated by M'hir et al. [6] where fungal protease from *R. oryzae* (supernatant preparation) was used to hydrolyse wheat gluten during long-time fermentation. Among fungi, the *Rhizopus* sp. is specific producers of extracellular proteases [10]. Also, *Rhizopus* strain was used to hydrolyse allergenic proteins from buckwheat [11].

Our early study on the crude extracellular-protease preparation from *Rhizopus oryzae*, showed this enzyme to be capable to increase proteolysis in wheat dough [6].

This study reports the statistical optimization of the medium for maximizing the production of the enzyme. Proteolytic activity by *Rhizopus oryzae* on gluten medium has been evaluated by using a 2^3 central composite design (CRD) and response surface methodology. The parameters investigated were gluten concentration, starch concentration and inoculums size.

MATERIALS AND METHODS

Microorganism and inoculums preparation

Rhizopus oryzae CH4, belonging to the Culture Collection of the Laboratory of Ecology and Microbial Technology was used in this study. The culture was grown on potato dextrose agar (PDA) plates at 30 °C for five days and the stock culture was maintained at 4 °C. To prepare spore inoculums, agar plates containing sporulative fungi were washed with sterile water to obtain a spore suspension. Spore count of the suspension was measured using the Malassez cell.

Culture media for protease production

Gluten (Sigma, G5004 Sigma #G5004, St Louis, MO, USA) was used as substrate for protease production. Into 500 ml Erlenmeyer flask, gluten was mixed with an enzyme production medium (100 ml), containing (g l⁻¹): KH₂PO₄ 2; KCl 5; FeSO₄ *7 H₂O 0.18; NaH₂PO₄ *H₂O 1.1; Na₂HPO₄ *12H₂O 8. The cotton plugged flasks were autoclaved at 121 °C for 20 min. After cooling the medium, they were inoculated with an appropriate volume of the spore suspension then kept on rotary shaker (200 rpm) at permanent conditions for 72 h. The supernatant (10,000 x g for 20 min at 4 °C) was used for the protease assay.

Protease activity was assayed with azocasein as substrate by the method previously described [6]. The method mentioned by Han et al. [12] was used to quantify protease activity with modification. Exactly 100 µl of *R. oryzae* CH4 supernatant and 300 µl of azocasein (2 %) (w/v) dissolved in citrate buffer at pH 5.2 were mixed (final volume 600 µl). The mixtures were incubated for 2 h at 45°C. The reaction was stopped by addition of 2 ml of 10 % TCA (trichloroaceticacid) (w/v) and the samples were held for 10 min in ice water before it was centrifuged (10,000 x g for 5 min). A unit of enzyme activity (UP) was defined as the amount of enzyme that produced an increase in absorbance at 440 nm of 0.01 after 2 h at 45°C. Arbitrary unit of proteolytic activity (UP ml⁻¹) was determined.

Experimental design

The factors studied were as follows: inoculums size, starch as carbon source, gluten concentration, each of which was assessed at five coded levels as shown in Table 1. For the three factors, the design was made up of a full 2^3 factorial design with its eight points augmented with four replications of the centre points (all factors at level 0) and the six star points, that is, points having for one factor an axial distance to the centre of $\pm \alpha$, whereas the other two factor are at level 0. The distance of the axial points was $\pm 1.68 (\alpha)$. A set of 18 experiments was carried out (Table 2) ($N = 2^K + 2K + n_0$, $K=3$, $n_0 = 4$). After running the experiments and measuring the activity levels, the experimental results of RSM were fitted with the response surface regression procedure using the following second order polynomial equation:

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

In this equation, Y is the predicted proteolytic enzyme production (response), X_i and X_j are the levels of the independent variables, β_0 is the intercept term, β_i is the linear coefficient, β_{ii} is the quadratic coefficient and β_{ij} is the interaction coefficient. Nemrodw software package was used for the regression analysis of the experimental data obtained [13]. The quality of the fit of the polynomial model equation was expressed by the coefficient of determination R^2 , and its statistical significance was checked by an F-test. The significance of the regression coefficient was tested by a t-test. The level of significance was given as *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$. Differences with p-value superior to 0.05 were not considered significant. For the validation of CCD, optimum conditions were fixed on the basis of the data obtained from the experimental design.

Protease Production in a Bioreactor

The verification of the statistical model for protease production was carried out in a 20-L bioreactor (Biolafite) (with a working volume of 12l). The optimized medium was sterilized in situ at 121°C for 20 min and inoculated with an appropriate amount of inoculum (6.6×10^6 spores ml^{-1}). Fermentation was carried out at 30 °C for 72 h with the pH controlled at 6.0. The impeller speed was initially adjusted to 97.5 rpm, and compressed sterile air was sparged into the medium at a constant rate of 1 vvm. 0.1% antifoam (v/v) was added to the reactor before sterilization. Samples were withdrawn and analyzed for protease production.

RESULTS

Based on earlier studies, gluten concentration, inoculums size and starch concentration were identified as the major factors affecting protease production by *Rhizopus oryzae* CH4. A total of 18 experiments with different combinations of the three variables were performed. The experimental design and the results are shown in Table 2. The highest protease activity (266.5 UP ml^{-1}) was observed at run number 12, where the factors gluten concentration, starch concentration and inoculums size were used at their levels 22.5 g l^{-1} , 30 g l^{-1} and 5×10^6 spores ml^{-1} respectively. This activity was about 3 fold higher than that observed at run number 6, where the related factors were used at high levels for X_1 (+1) and X_3 (+1) and low level for X_2 (-1).

The protease production (Y) by *Rhizopus oryzae* can be expressed in terms of the following regression equation:

$$Y = 261.233 - 19.138 X_1 + 27.627 X_2 + 14.075 X_3 - 28.721 X_1^2 - 18.733 X_2^2 - 42.332 X_3^2 + 14.125 X_1 X_2 + 2.750 X_1 X_3 + 33.750 X_2 X_3;$$

where X_1 =gluten concentration, X_2 = Starch concentration, X_3 = inoculums sizes

The regression coefficients and the analysis of the variance (ANOVA) indicate the high significance of the model (Table 3). The high R^2 value 0.981 showed the good agreement between the experimental results and the theoretical values predicted by the model [14]. The R^2 value is always between 0 and 1. The closer the R^2 is to 1.0, the stronger the model and the better it predicts the response [15]. The value of R^2 indicated that only 1.9 % of the total variations were not explained by the model. The value of the adjusted determination coefficient ($\text{Adj } R^2 = 0.959$) was also very high to advocate for a high significance of the model [15]. A lower value of coefficient of variation ($\text{CV} = 4.28\%$) showed the experiments conducted were precise and reliable [16].

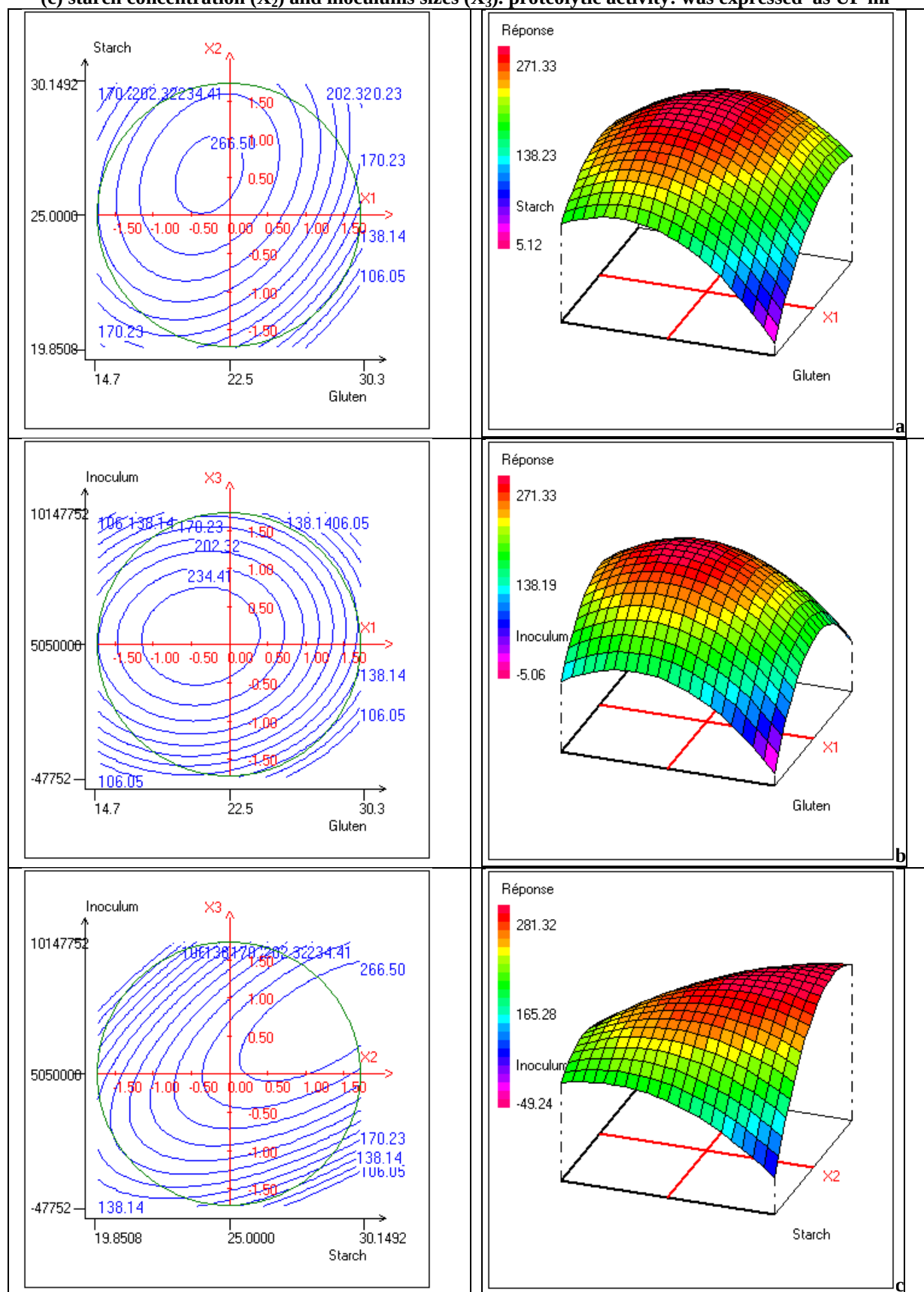
The significance of each coefficient was determined by P-values which were listed in Table 4. The ANOVA analysis of the optimization study indicated that X_1 , X_2 , X_1^2 , X_3^2 , $X_2 X_3$ were more significant ($p < 0.001$) than the effect of other variables. Gluten concentration had a negative effect on protease production, however, starch and inoculums size exerted a positive influence. Starch concentration was the most important factor affecting protease production ($\beta_2 = 27.627$, $P < 0.001$). Negative quadratic effects were obtained for gluten, starch and inoculum size. Positive interaction was shown between gluten and starch ($P < 0.01$), and between starch and inoculums size at the $P < 0.001$ probability level. The coefficient of the interaction $X_1 X_3$ was found to be not significant.

The 2D contour plot and 3D response surface were generally the graphical representation of the regression equation. Fig 1 represented the 3D contour plots for the optimization of medium components of protease production. Each figure presented the effect of two variables on the production of protease, while other two variables were held at zero level. Maximum protease production (266.5 UP ml^{-1}) was achieved at gluten 22.5 g l^{-1} , starch 30 g l^{-1} and inoculums size 5×10^6 spores ml^{-1} . As is shown in Fig 1 A, a linear increase in protease secretion was observed when gluten and starch concentrations were increased. Then a negative effect was observed when gluten concentration increased. In Fig 1B protease production could not increase with increasing gluten or inoculums levels. Fig 1C depicts the interaction of inoculums size and starch concentration where the shape of the response surface indicates positive interaction between these two factors. Maximum enzyme production was recorded in the middle levels of both the factors while further increase in the levels resulted in a gradual decrease in yield.

DISCUSSION

The crude enzyme of *Rhizopus oryzae* CH4 strain showed in previous study gluten hydrolysis [6]. The improvement of microbial protease production is the purpose of several investigations. In general, no defined medium has been carried out for the production of proteases from different microorganisms; each strain has its specific required conditions for maximum enzyme production. The use of statistical models to optimize culture medium components and conditions has increased in present-day biotechnology, due to its ready applicability and aptness [17, 18].

Figure 1. Contour plots and response surface plot for protease production showing the interactive effects of : (a) the gluten (X_1) and starch concentrations (X_2), (b) gluten concentration (X_1) and inoculums sizes (X_3), and (c) starch concentration (X_2) and inoculums sizes (X_3). proteolytic activity: was expressed as UP ml⁻¹



In the previous study, the significant variables necessary for enhanced protease production were selected using the fractional factorial design (data not shown). Among the three significant variables selected, starch concentration, inoculums size and gluten concentration were found to influence enzyme secretion. Complex carbon sources like starch constitute better substrates for protease production than simple sugars, such as glucose, which can induce catabolic repression [17, 19].

In the literature, only the report of Tunga et al. [20] used factorial design technique to optimize a culture medium for the production of protease by *Rhizopus* sp. Whereas, *Rhizopus* sp. are well known to produce high proteolytic enzyme [21-23]. Tunga et al. [20] has been investigated three inducers: biotin, metal ion (CaCl_2) and plant hormone to maximize protease production. Gluten was not usually used as nitrogen source due to high cost. In this study, we investigated on gluten to induce protease production by this fungus to produce protease able to breakdown it. This protein was complex and resist to proteolysis. Protease production by *Rhizopus* spp. grown in media containing cereal storage proteins has not been studied previously. Only the study of Pekkarinen et al. [24] induce protease production by *Fusarium culmorum*, which grown in gluten-containing media at a concentration 8 g l^{-1} . This strain produced 1400 U of the enzyme, as estimated by the azogelatin method. In the study of Shivakumar [25], Wheat flour used as agro industrial supported a high titre of protease activity. Application of different methods and the definition of enzyme units to estimate the enzyme by various authors using different substrates such as casein, haemoglobin, methylcoumarylaminoacid (MCA), azocasein or gelatin make the comparison difficult. However, our estimations have shown that the protease activity appears to be as good [6].

Table 1. Experimental codes, ranges and levels of the independent variables of the 2^3 factorial design

Symbol code	Independent variables	Levels				
		-1.68	-1	0	+1	+1.68
X_1	Gluten (g l^{-1})	15	18	22.5	27	30
X_2	Starch (g l^{-1})	20	22	25	28	30
X_3	Inoculum size (spores ml^{-1})	10^5	2.10^6	5.10^6	8.10^6	10^7

Table 2. Experimental design and results of central composite design for the optimization of protease production

Run	X_1	X_2	X_3	Proteolytic activity (UP ml^{-1})	
				Experimental	Predicted
1	-1	-1	-1	190.00	199.508
2	1	-1	-1	120.00	127.483
3	-1	1	-1	148.50	159.011
4	1	1	-1	134.00	143.486
5	-1	-1	1	150.00	154.659
6	1	-1	1	90.00	93.633
7	-1	1	1	242.50	249.162
8	1	1	1	240.00	244.637
9	$-\alpha$	0	0	224.00	212.184
10	$+\alpha$	0	0	156.00	147.812
11	0	$-\alpha$	0	170.00	161.786
12	0	$+\alpha$	0	266.50	254.711
13	0	0	$-\alpha$	133.00	117.826
14	0	0	$+\alpha$	170.00	165.170
15	0	0	0	262.000	261.233
16	0	0	0	257.000	261.233
17	0	0	0	260.000	261.233
18	0	0	0	262.500	261.233

Average value of duplicate determination except design points 15 to 18

Concerning inoculums sizes, an increase in the enzyme activity was recorded with increasing concentration of spores in inoculums until a limit (about 5×10^6 spores ml^{-1}) over which the enzyme activity decreases. These finding are in line with several reports [20, 26]. An increase in the size inoculums did not reveal increased protease activity. For *Rhizopus oryzae* NRRL 21498, the optimum spore concentration required for maximum protease activity was about 2×10^5 spores g^{-1} of wheat bran.

Validation of the model in shake flask culture

The suitability of the model equation for predicting the optimum response value was tested using the recommended optimum conditions. When optimum values of independent variables (gluten concentration 22.1 g l^{-1} , starch concentration 27 g l^{-1} , inoculums size 6.6×10^6 spores ml^{-1}) were incorporated into the regression equation, 279.37 UP

ml⁻¹ was obtained whereas experiments at optimum conditions gave a protease production of 270.1 UP ml⁻¹. Thus, predicted values from fitted equations and observed values were in very good agreement.

Verification of Model in a 20-L Bioreactor

The conditions obtained as optimum through RSM were finally verified in a 20-L bioreactor. Protease production was 258.2 UP ml⁻¹ was obtained between 60 and 72 h of growth.

Table 3. Analysis of variance for protease production

Source of variation	Sum of squares	Degrees of freedom	Mean square	Ratio	Significance
Regression	5.73468E+0004	9	6.37187E+0003	44.8736	***
residuals	1.13597E+0003	8	1.41996E+0002		
Total	5.84828E+0004	17			

*** Denotes significant at 0.1 % level ; $R^2 = 0.981$; $CV = 4.28\%$; $Adj R^2 = 0.959$; $Pred R^2 = 0.855$

Table 4. Test of significance for regression coefficient

Model term	Coefficient estimate	Standard error	Significance %
Intercept	261.233	5.949	***
X ₁	-19.138	3.224	***
X ₂	27.627	3.224	***
X ₃	14.075	3.224	**
X ₁ ²	-28.721	3.350	***
X ₂ ²	-18.733	3.350	***
X ₃ ²	-42.332	3.350	***
X ₁ X ₂	14.125	4.213	**
X ₁ X ₃	2.750	4.213	53.8%
X ₂ X ₃	33.750	4.213	***

The level of significance was given as *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$

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