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POULTRY DROPPING BASED BIODIGESTED SLURRY FOR PROTEASE PRODUCTION BY *Humicola fuscoatra* MTCC 1409: OPTIMIZATION AND KINETIC STUDY

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First order kinetics

Gibbs free energy

Humicola fuscoatra MTCC 1409

Protease

Poultry dropping based biodigested slurry

ABSTRACT

Protease is one of the most important groups of commercially produced enzymes. This study was aimed at the optimization and kinetics of protease production from poultry dropping based biodigested slurry by *Humicola fuscoatra* MTCC 1409. Four significant variables (pH, temperature, slurry concentration and inoculum concentration) were considered for optimization both by one variable at a time approach and response surface methodology. The maximum protease production in the poultry dropping based biodigested slurry was (531±1.37 U g^{-1}) under the optimum conditions of pH (5), temperature (40°C), slurry concentration (25%) and inoculum concentration (10%). The protease production was found to be 3.38 fold higher under optimized conditions as compared to the non-optimized ones. The thermal inactivation of protease produced from biodigested slurry was investigated kinetically within temperature range of 30-70°C. The irreversible inactivation was well described by first order kinetics with k values increasing between 0.0028 to 0.0071 min^{-1} and $t_{1/2}$ decreasing from247.70 to 98.10 mins. At higher temperature, there was significant decrease in residual activity. The activation energy, enthalpy, Gibbs free energy and entropy range calculated on the basis of residual activity experiments conducted at temperature range of 30-70°C was found to be 21.29, 18.44 to 18.78 kJ mol^{-1}, 89.01 to 98.47 kJ mol^{-1} and -0.23334 to -0.23181 kJ mol^{-1} respectively, suggesting the thermostability of enzyme. This is first report on optimization, kinetics and determination of thermodynamic parameters of protease production by *Humicola fuscoatra* MTCC 1409 from poultry dropping based biodigested slurry.

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1 Introduction

Proteases (EC 3.4), hydrolyzing proteins to short peptides or free amino acids (Barrett & McDonald, 1986), account for nearly 60% of the industrially important enzyme market. These are used in many industries like detergents, food processing, animal nutrition, pharmaceuticals, textiles and paper (Kamath et al., 2010). Recently, researchers developed interest in fungal proteases due to high diversity, broad substrate specificity and stability under extreme conditions, besides producing acidic, neutral and alkaline proteases (Tremacoldi et al., 2004). Moreover fungi can be grown on cheaper substrates over a wide range of pH (4.0-11.0) (Murthy & Naidu, 2010) and produce extracellular enzymes, that can be easily recovered from fermenting broth by simple filtration (Chandrasekaran et al., 2015).

Poultry industry waste consists of mainly a mixture of poultry droppings, bedding material (e.g. wood shavings or straw), dead birds, waste feed, broken eggs and feathers removed from poultry houses (Kelleher et al., 2002). Poultry litter has high pollutant load so, its direct application on land may lead to nutrient leaching, spread of pathogens, production of phytotoxic substances, eutrophication of water bodies, and other air pollutants (Costa et al., 2012), No doubt, its disposal is of great concern but being organic in nature, the poultry droppings can be utilized for biogas generation.

Poultry droppings had been found to generate more biogas than piggery and cattle wastes (Rao et al., 2011). Biogas production from poultry droppings helps us to meet our energy sources. The anaerobic digestion of poultry droppings yields approximately 60% methane, 38% carbon dioxide, and mixture of water vapors, ammonia and hydrogen sulphide (Bolan et al., 2010). The use of agro-industrial waste, as cultivation media, is a matter of great interest, as this may help to decrease the costs of enzyme production (Singh et al., 2009). After biogas production, safe disposal of slurry is a big problem as it is very difficult to transport viscous material to the fields. Also, its direct application in the soil, forms a slow degradable layer on the soil surface, which is not desirable. Poultry dropping based biodigested slurry contains large amount of proteins, nitrates and amino acids, that can be utilized as substrate for protease production.

Thermodynamic studies can provide information about the thermostability of enzymes at the operating temperature. The enzyme undergoes a first order kinetics reaction, which is responsible for its irreversible denaturation and is expressed in terms of its half life ($t_{1/2}$). The activation energy and change in Gibbs free energy, enthalpy and entropy between the folded and unfolded states of enzyme are to describe denaturation thermodynamics (Saqib et al., 2010). The activity and thermostability of enzymes are important parameters to determine the economic feasibility in industrial processes. High stability is generally considered an economic advantage because of its reduced enzyme turnover (Vielle & Zeikus, 2001).

Various factors like temperature, pH, slurry concentration and inoculum concentration can be optimized by Response Surface Methodology using Statgraphics Centurian XVI.I software. The main advantage of response surface methodolgy is the reduced number of experimental runs needed to provide sufficient information for statistically acceptable results. No reports are available on protease production from *Humicola fuscoatra* MTCC 1409 using poultry dropping based biodigested slurry. Thus, the study was designed to optimize the cultural conditions for protease production by *H. fuscoatra* MTCC 1409 using poultry dropping based biodigested slurry which will be a cheaper substrate for decreasing the cost of enzyme production.

2 Materials and Methods

2.1 Procurement of substrate and culture

The poultry droppings, collected from the Guru Angad Dev Veterinary and Animal Sciences, University (GADVASU), Ludhiana (India) were used in lab scale 10 liter biogas digester set up at Biogas Laboratory, Punjab Agricultural University (PAU), Ludhiana (India).

Standard culture of *H. fuscoatra* MTCC 1409 was procured from Microbial Type Culture Collection (MTCC), Institute of Microbial Technology, Chandigarh (India) and was maintained on potato dextrose agar slants at 40±2ºC by monthly transfers. The culture was stored at 4ºC after sub-culturing.

2.2 Qualitative enzyme estimation

The fungal culture was studied qualitatively for protease enzyme production by clearance zone method (Saran et al., 2007). *H. fuscoatra* MTCC 1409 was point inoculated on the Petri plates containing skimmed milk medium and incubated at 40°C. Plates were flooded with tannic acid (10%) after luxuriant growth of fungi and were observed for the formation of clear zone around the colony which represents the production of proteases by the fungus. Potency index was calculated as given below:

$$\text{Potency index} = \frac{\text{Area of clearance zone (cm}^2\text{)}}{\text{Area of colony (cm}^2\text{)}}$$

2.3 Protease production from biodigested slurry through fermentation

For protease production, 50 ml poultry dropping based biodigested slurry was taken in Erlenmeyer flasks (250 ml) and diluted by using 50 ml distilled water. Flasks were inoculated with the 10^6 spore/ml (measured by haemocytometer) of *H. fuscoatra* MTCC 1409, incubated for 4 days at 40°C. The enzyme was extracted by centrifuging the incubated slurry at 10,000 rpm for 20 minutes at 4ºC and supernatant was used for estimation of

protease activity by spectrophotometric method. The experiment was performed in triplicates. The required spore concentration was obtained by using the formula given below:

$$X = \frac{\text{Required Spore Concentration} \times \text{Final Volume Needed}}{\text{Hemocytometer count}}$$

Where,

X = volume of spores suspension to be added

2.4 Quantitative estimation of protease

Protease estimation was carried out according to the method described by Enyard (2008). Enzyme extract (0.1 ml) was taken in triplicate test tubes and mixed with 0.9 ml of distilled water. Then, 5 ml of 0.65% casein solution was added. The mixture was incubated at 37°C for 10 min. After incubation, 5 ml of tri-chloroacetic acid (TCA) was added and the mixture was incubated at 37°C for 30 min. To measure protease produced during this reaction, 5 ml of Na_2CO_3 solution (500mM) was added followed by immediate addition of 1 ml Folin phenol reagent. Mixture was kept at 37ºC for 30 min. Control experiment devoid of substrate, was run simultaneously. The % light absorbance was recorded at 660 nm in a UV-VIS spectrophotometer (Hitachi UV-VIS U-2800). A standard curve for protease activity was prepared under same conditions as described above using standard solution of L-tyrosine in the concentration range of 0.01 to 1.0 g. The corresponding enzyme activity was calculated from the standard curve.

2.5 Optimization of enzyme production by 'one variable at a time approach'

The protease enzyme production by *H. fuscoatra* MTCC 1409 was first optimized by 'one variable at a time approach' to find the most important factors that affect its production. The effect of each variable like incubation temperature (35°C, 40°C and 45°C), pH (5, 6 and 7), slurry concentration (25%, 50% and 75%) and inoculum concentration (5%, 10% and 15%) on enzyme production was studied. Enzyme activity of all the triplicates sets was measured on 4^{th} day.

2.6 Statistical optimization of protease production using RSM

Statistical optimization via multilevel factorial design using a response surface methodology (RSM) approach was used for protease production. Four factors selected by 'one variable at a time' approach, were used in designing the experiment: slurry concentration (25-75%), inoculum concentration (5-15%), pH (5-7) and temperature (35-45°C). The triplicate flasks with biodigested slurry were inoculated with inoculum of *H. fuscoatra* MTCC 1409, incubated at required temperature as per the sets formed by response surface methodology software. Table 1 illustrates independent variables with their coded and actual values.

Table 1 Independent variables with their coded and actual values

Factors	Units	Coded Levels		
		-1	0	+1
Inoculum Concentration	%	5	10	15
Slurry Concentration	%	25	50	75
pH	-	5	6	7
Temperature	°C	35	40	45

The following second order polynomial equation was used to determine the relationship between the response and the independent variables

$$y = \beta_0 + \Sigma \beta_i x_i + \Sigma \beta_{ii} x_i^2 + \Sigma \beta_{ij} + x_i x_j$$

Where,

y is the predicted response, β_0 is the interception co-efficient, β_i is the linear coefficient, β_{ii} is the quadratic coefficient, β_{ij} is the interaction coefficient.

For optimization, the analysis of variance (ANOVA) for the overall effect of four factor variables on the response variable according to fitted model was done using software Statgraphics Centurian XVI.I and the least significant factor affecting the response variable was selected. Response surface methodology (Multilevel Factorial Design) was used for multiple regression analysis of the experiment and F test was employed to evaluate the statistical significance of quadratic polynomial equation. The performance of regression equation was evaluated by determination coefficient of correlation (R^2). The contour plots were generated to study the interaction of various factors taken for protease production. The various combinations obtained, under which experiments were performed are given in Table 2.

2.7 Validation of model

The protease production experiment from *H. fuscoatra* MTCC 1409 was conducted under optimized conditions i.e. biodigested slurry of 25% concentration, inoculum concentration of 10% and pH of 5 obtained through RSM and all flasks were incubated at optimized temperature i.e. 40ºC for 4 days. The observed value from validation experiment was compared with the value obtained through RSM and the predicted one. The crude enzyme was analyzed for protein content and protease activity.

2.8 Estimation of kinetic parameters

Enzyme kinetics was studied using first order reaction that describes the irreversible thermo-inactivation process of protease enzyme given below (Souza et al., 2015):

Table 2 Protease activity of experimental runs by Response Surface Methodology (RSM)

S. No.	Inoculum Concentration (%)	Slurry Concentration (%)	Temperature (°C)	pH	Protease Activity (Ug^{-1})		Desirability	
					Observed	Predicted	Observed	Predicted
1	-1	+1	-1	-1	150±1.98	160.77	0.61	0.60
2	0	0	+1	0	296±1.74	306.46	0.78	0.80
3	+1	0	+1	+1	195±1.23	194.80	0.65	0.65
4	0	+1	0	-1	290±1.01	291.65	0.72	0.72
5	+1	+1	-1	+1	182±1.94	183.74	0.73	0.74
6	0	-1	-1	0	383±1.78	384.26	0.69	0.70
7	0	0	-1	+1	150±1.08	150.87	0.62	0.61
8	-1	+1	+1	-1	196±1.05	191.99	0.73	0.73
9	0	0	-1	-1	320±1.07	308.41	0.70	0.70
10	-1	-1	0	0	172±1.45	174.17	0.61	0.62
11	0	+1	0	+1	420±1.56	413.69	0.77	0.77
12	0	0	0	-1	395±1.42	400.29	0.83	0.85
13	+1	-1	+1	0	287±1.37	278.13	0.72	0.72
14	-1	0	+1	0	161±1.74	160.15	0.65	0.64
15	-1	0	-1	-1	148±1.87	142.52	0.67	0.67
16	0	-1	0	-1	**531±1.37**	**532.31**	**0.96**	**0.93**
17	+1	+1	0	0	207±1.51	199.99	0.73	0.71
18	+1	0	-1	0	287±1.48	291.84	0.67	0.67
19	+1	+1	+1	+1	110±1.36	116.29	0.68	0.69
20	+1	-1	0	-1	380±1.32	386.13	0.73	0.74

Data is the mean of three replications or determination ; ± represents standard error

$$A/A_o = \exp(-kt) \quad (1)$$

Where A/A_o is the residual activity at the time t (min), and k (min^{-1}) is the inactivation rate constant at resolute temperature. The inactivation rate constant (k) can be calculated by non-linear regression analysis.

The enzyme half-life ($t_{1/2}$) was defined as the time after which (A) initial enzyme activity was reduced to one half the initial value and measured as:

$$t_{1/2} = \frac{\ln(2)}{k} \quad (2)$$

D value is the time needed to reduce the initial enzyme activity by 90%. Algebraically expressed as:

$$D = \frac{\ln(10)}{k} \quad (3)$$

The other thermodynamic parameters of protease inactivation were estimated by Arrhenius law:

$$\ln(k) = \ln(C) - \frac{Ea}{R*T} \quad (4)$$

Where C is the Arrhenius constant, E_a (kJ mol^{-1}) the activation energy, R(8.31 kJ mol^{-1}) the universal gas constant and T is the absolute temperature. The E_a can be estimated by the slope of linear regression analysis of the natural logarithm of rate constant versus the reciprocal of the absolute temperature.

Using estimated value of E_a, the activation enthalpy (ΔH) for each temperature was calculated by:

$$\Delta H = E_a - R*T \quad (5)$$

The free energy of inactivation (ΔG) was determined according to the expression:

$$\Delta G = -R * T * \ln\left(\frac{k*h}{K_B*T}\right) \quad (6)$$

Where h is 6.6262 x 10^{-34} (J s) is the Planck's constant, K_B is 1.3806 x 10^{-23} (J/K) is the Boltzmann's constant, and k (s^{-1}) the inactivation rate constant of each temperature. From Eq (5) and (6) it the activation entropy (ΔS) was calculated by:

$$\Delta S = \frac{\Delta H - \Delta G}{T} \quad (7)$$

3 Results and Discussion

3.1 Qualitative protease estimation: After flooding with tannic acid, clear zone was observed with the potency index of 1.81 as shown in Figure 1. The tannic acid increases the colour intensity of plate, because of the precipitation of unhydrolyzed protein in the plate, thus improving the contrast between the intact zones and

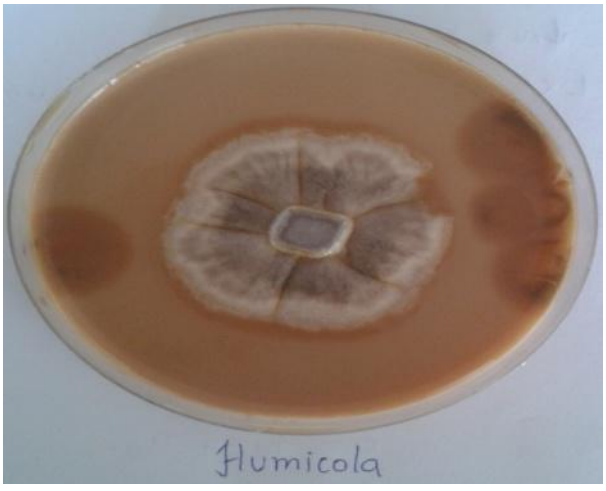

Figure 1 Zone on skimmed milk agar after flooding with tannic acid with potency index of 1.81

the enzymatic lyses zones of the substrate (Gnanadoss et al., 2011).

3.2 Optimization by 'one factor at a time approach'

H. fuscoatra MTCC 1409 grew well in poultry droppings based biodigested slurry by forming a thick grey sheath on the surface of the medium. Maximum protease production (159 Ug^{-1}) was found at 40°C which is optimum temperature for its growth. However, with the increase and decrease of temperature the enzyme activity was decreased (Figure 2a). Reduction in enzyme activity was observed both at above and below optimum level of inoculum concentration (Figure 2b). The inoculums concentration of 10% yielded maximum (132 Ug^{-1}) protease production. This might be due to the fact that lower inoculum concentration is insufficient for enzyme production and higher inoculum concentration caused competition for nutrients (Dar & Phutela, 2015). Maximum enzyme activity (149 Ug^{-1}) was observed at the 25% slurry concentration (Figure 2c) which is in line to the results of Lakshmi et al. (2014) who found maximum protease activity of 961.25 Uml^{-1} after 72 hrs using 10% substrate (Wheat bran) concentration from *Humicola grisea* . The enzyme yield was reduced at lower and higher levels of slurry concentration. Medium pH being the important factor was also optimized for maximum enzyme production. It was found that the maximum protease production (145Ug^{-1}) was at pH 5 (Figure 2d). Similar results were also reported by Negi & Banerjee (2006); Oyeleke et al. (2010) and Gnanadoss et al. (2011).

3.3 Statistical optimization of cultural conditions for protease production by RSM

The effect of four factors spore concentration (5, 10 and 15%), incubation temperature (40, 45 and 50°C), pH (5, 6 and 7) and slurry concentration (25, 50 and 75%) at different levels on the response variable, "protease activity" was studied using Multilevel Factorial Design (MFD). As suggested by RSM, 20 experimental runs were performed and results are shown in Table 2.

The analysis of variance (ANOVA) and analysis of the experimental results (Table 3) indicated that the developed model for each measured response had a good lack of fit. The coefficient of determination (R^2) was 0.997 for protease production. Figure 3 indicating good correlation between input factors and their responses and hence suggesting significant correlation between the model and measured data. The R-squared statistics indicated that the model as fitted explains 99.70 % of the variability in protease. The adjusted R-squared statistic, which is more suitable for comparing models with different numbers of independent variables was 98.89 %. The standard error of the estimate showed the standard deviation of the residuals to be 12.00. Experimental

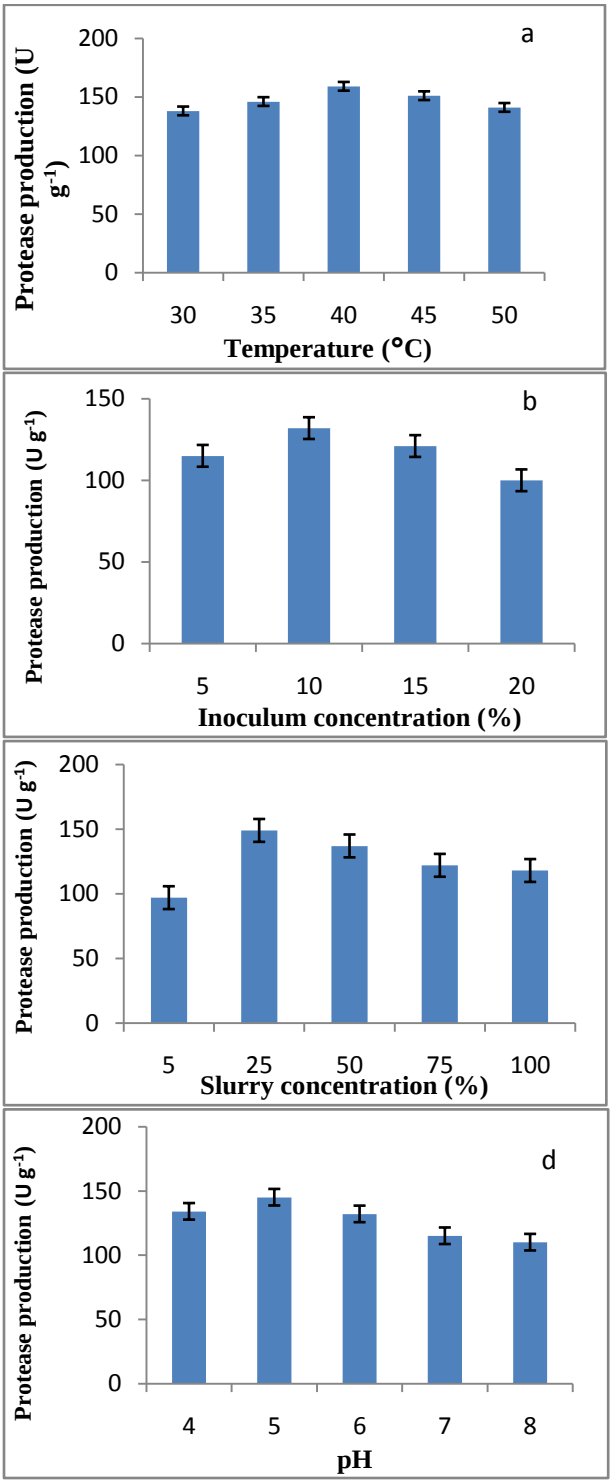

Figure 2 (a) Effect of temperature on protease production (b) Effect of inoculum concentration on protease production (c) Effect of slurry concentration on protease production. (d) Effect of pH on protease production

Table 3 Analysis of variance for protease activity

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
A:Inoculum Concentration	19371.8	1	19371.8	134.31	0.0001
B:Slurry Concentration	6990.24	1	6990.24	48.47	0.0009
C:Temperature	988.526	1	988.526	6.85	0.0472
D:pH	1117.06	1	1117.06	7.74	0.0388
AA	83486.9	1	83486.9	545.83	0.0004
AB	35742.3	1	35742.3	217.81	0.0002
AC	15744.6	1	15744.6	109.16	0.0001
AD	21526.6	1	21526.6	149.25	0.0001
BB	325.532	1	325.532	2.26	0.1933
BC	17.8802	1	17.8802	0.12	0.7391
BD	14409.2	1	14409.2	99.90	0.0002
CC	27751.8	1	27751.8	164.41	0.0003
CD	7861.53	1	7861.53	54.51	0.0007
DD	12079.2	1	12079.2	83.75	0.0003
Total error	721.164	5	144.233		
Total (corr.)	247977.	19			

R-squared	99.7092%
R-squared (adjusted for d.f.)	98.8949%
Standard Error of Est	12.0097
Mean absolute error	4.84779

results were very close to predicted ones for protease production as given in Table 2.

ANOVA for the four variables indicated that the protease production could be described well by Multilevel Factorial Design model with a relatively high coefficient of determination. The effect of independent variables inoculum concentration (p=0.0001), slurry concentration (p=0.0009), temperature (p=0.0472) and pH (p=0.0388) were significant as there p value was less than 0.05. All the interactions were significant except BB (p=0.1933) and BC (p=0.7391) as p value >0.05. Pareto chart also showed similar results (Figure 4). Pareto chart revealed that while increase in inoculum concentration exerted a positive effect on protease activity whereas increase in slurry concentration, pH or temperature showed negative effect i.e. decreased protease activity.

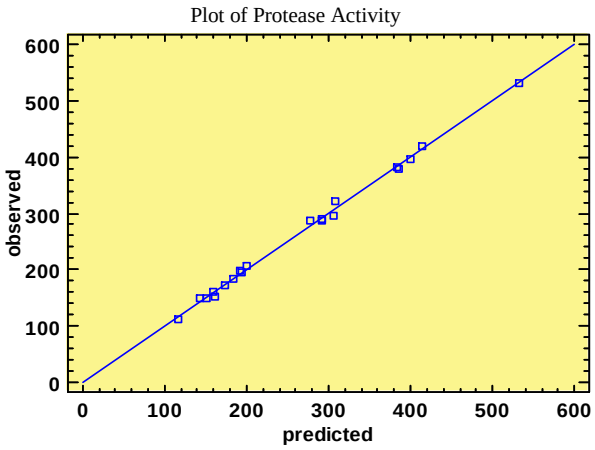

Figure 3 Experimental Vs predicted results of protease production

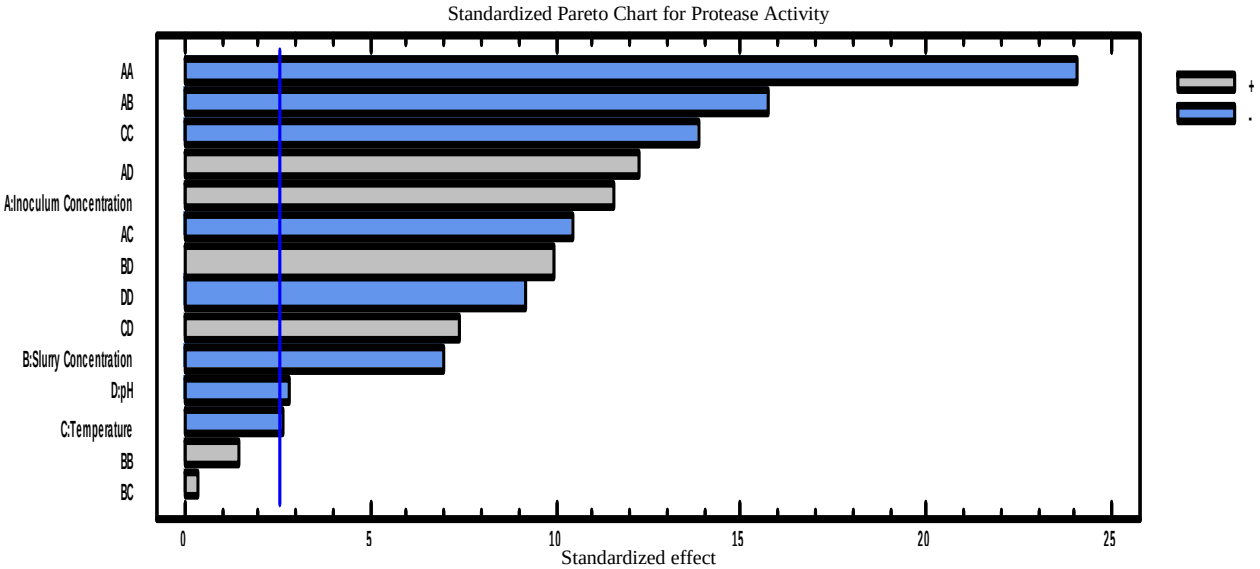

Figure 4 Pareto Chart

Enzyme production increases with increase in temperature till 40°C, then decreases drastically which could be because of enzyme denaturation at higher temperature (Badhe et al., 2016).

Similarly, optimum pH for protease production was found to be 5. There are many reports on acidic range of optimum pH from different fungi *Rhizopus oryzae* (pH 5.5) (Kumar et al., 2005) and

Aspegillus niger (pH 4) (Siala et al., 2012). The regression equation of the fitted model for protease is:

-10239.2+159.27× A - 12.73× B + 483.47×C + 219.17×D – 7.10 × AA – 1.03× AB -3.44× AC + 30.46× AD +0.01×BB + 3.14× BD – 6.57× CC +12.25× CD – 99.04× DD

Where A = inoculum concentration; B = slurry concentration; C = temperature; D= pH

3.4 Contour plots for various combination of variables

In order to examine the interaction between different variables and their impact on each other, contour plots were developed and studied. Contour plots are generally aimed to describe the function of two factors at a time, keeping other factors constant and hence helpful in understanding the interaction between two factors. The contour plots are shown in Figure 5, 6, 7, 8, 9 and 10. Interactions among various variables can be easily explained by keenly observing the contour plots because, as a rule, the more elliptical the shape of the contour, the better is the interaction between the two variables while circular shape indicates least interaction between the variables. From our results, shown in Figure 5, 6, 7, 8, 9 and 10, it is clear that except one slurry concentration versus temperature interaction, all others are significant.

3.4.1 Slurry concentration verses inoculum concentration

Both slurry and inoculum concentration is significant as its *p* value is 0.0002. Figure 5 clearly shows that increase in inoculum concentration increases the protease activity but more increase in slurry concentration decreases the activity. This could be because as the slurry concentration increases, substrate size increases but inoculum concentration remains lowcomparatively to substrate size.

3.4.2 Temperature verses inoculum concentration

Temperature and inoculum concentration have significant effect on the protease production ($p < 0.05$). Figure 6 shows that highest protease activity was found at 39-41°C temperature and 5-9% inoculum concentration. With increase in inoculum concentration to 15% and temperature to 45°C, protease activity decreased. This could be due to the fact that the optimum temperature for the growth of *H. fuscoatra* MTCC 1409 is 40±2°C and increase in inoculum concentration will lead to decrease in nutrients for growth of fungi (Raj et al., 2012).

3.4.3 pH verses inoculum concentration

pH and inoculum concentration have a prominent effect on the production of protease production as was observed during the study ($p = 0.0001$, which is less than 0.05). Figure 7 indicates that decrease in pH does not affect the activity significantly but increase in inoculum concentration decreases the activity. This suggests that extracellular enzyme is acidic in nature and with increase in pH, activity decreases. With the increase of inoculum concentration, decrease in activity is probably due to limitation in nutrients for growth (Suganthi et al., 2013).

3.4.4 Temperature verses slurry concentration

Temperature and slurry concentration showed non significant interaction, $p = 0.7391$. Figure 8 shows that protease activity increased with the increase in temperature from 35-41°C and was highest at 25% slurry concentration. Further increase in temperature and slurry concentration showed the decrease in protease activity. With increase in temperature there is decrease in activity as the optimum temperature for the growth of *H. fuscoatra* MTCC 1409 is 40±2°C, so further increase in temperature decreases the viability of the fungus and leads to decrease in activity.

3.4.5 pH verses slurry concentration

pH and slurry concentration showed significant interaction, $p = 0.0002$. Figure 9 illustrates that maximum protease activity was at 25% slurry concentration and 5.4-5.8 pH. Increase in slurry concentration and pH, decreased the protease activity. Many extracellular proteases are active in acidic environments, and hence increase in pH decreases its activity (Siala et al., 2012; Chandrasekaran et al., 2015). Increase in slurry concentration decreased the enzyme activity as inoculum level is insufficient to utilize the increases slurry for enzyme production.

3.4.6 pH verses temperature

Results show that pH and temperature are also important factors for protease production and significantly interact with each other ($p<0.05$). Figure 10 indicates that when the temperature was between 39-41°C and pH 5-5.4, maximum protease activity was observed but increase in pH to 7 and temperature to 45°C decreased the protease production (Sumantha et al., 2006; Vishwanatha et al., 2010).

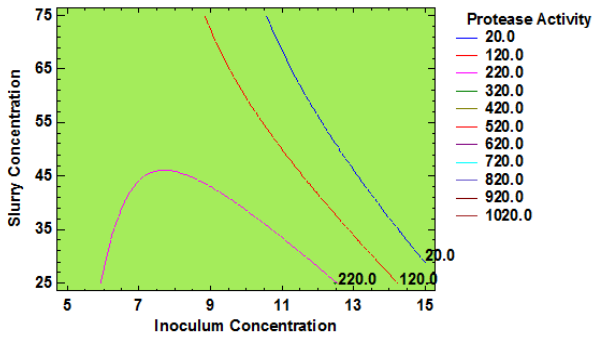

Figure 5 Contour plot between Slurry concentration and inoculum concentration

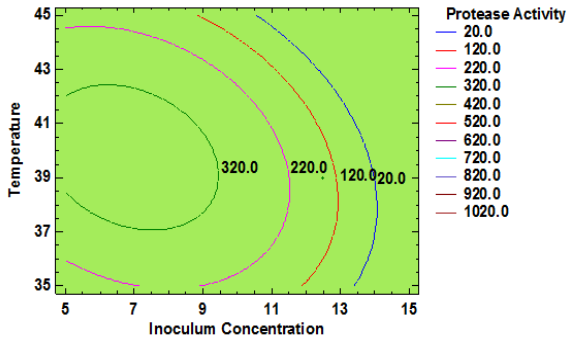

Figure 6 Contour plot between temperature and inoculum concentration

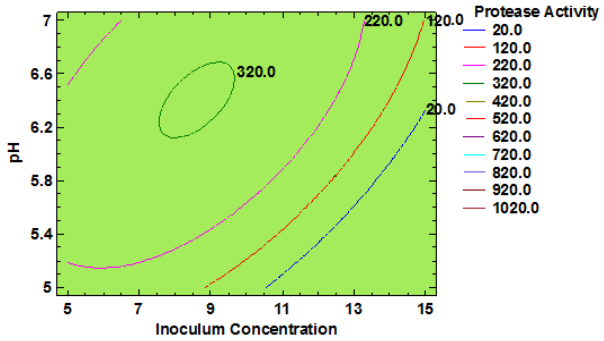

Figure 7 Contour plot between pH and inoculum concentration

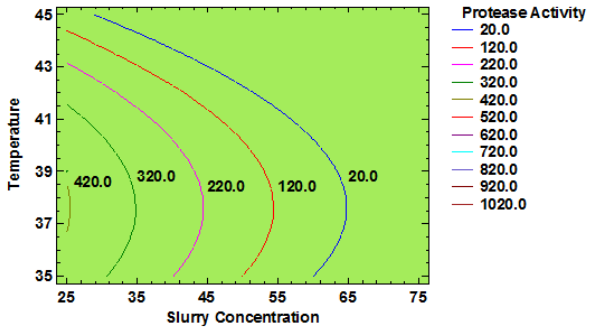

Figure 8 Contour plot between temperature and slurry concentration

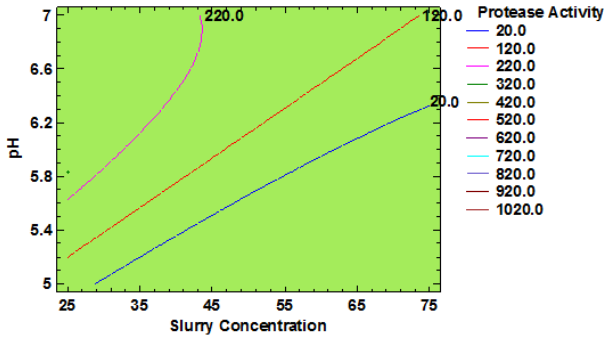

Figure 9 Contour plot between pH and slurry concentration

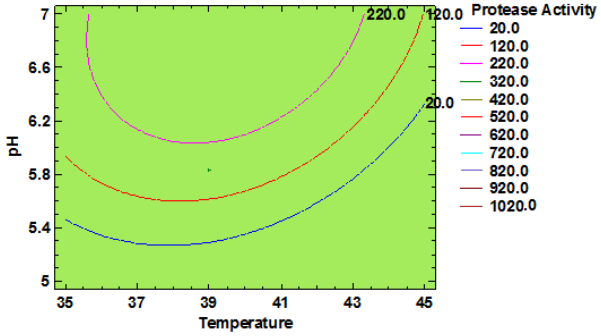

Figure 10 Contour plot between pH and temperature

Table 4 Validation of protease produced under best optimized conditions

S. No.	Inoculum Concentration (%)	Slurry Concentration (%)	Temperature (°C)	pH	Protease Activity (Ug^{-1})		
					Validated	By RSM	Predicted
1	10.0	25.0	40.0	5.0	527±1.37	531±1.28	532.31

3.5 Validation of protease production under optimized conditions

Results as given in Table 4, indicated the close relationship between the observed experimental (RSM) (531Ug^{-1}), predicted (532.31 Ug^{-1}) value and the response value from validation experiment (527 Ug^{-1}). This shows the validity and acceptability of the model for the optimization of inoculum concentration, slurry concentration, pH and temperature for protease production

from *H. fuscoatra* MTCC 1409. Therefore, this can be further used for large scale production.

3.6 Kinetic parameters

The half-life ($t_{1/2}$) which is the time required for the enzyme activity to drop down to 50% of initial value at a given temperature is significant on industrial point of view because higher the half life of enzyme, the higher is its thermostability. Figure 13 shows the relation between the Decimal reduction time (D) and temperature on enzyme production at different temperature. Results given in Table 5 illustrate that $t_{1/2}$ decreased and the specific rate of the first order protease thermo in-activation (k^{-1}) increased with increase in temperature. The value of k^{-1}(0.0031 min^{-1}) and $t_{1/2}$ (224.40 min) at 40°C, which is the optimum temperature for protease production, was obtained.

The activation energy (E_a, 21.29 kJ mol^{-1}) of protease denaturation was obtained from Log plot of ln(k) vs 1/T (Figure 12), which is lower than reported for protease from *Aspergillus fumigates* (E_a, 69.00 kJ mol^{-1}) as reported by Martinez et al. (2011). Very large variability of E_a has been reported in previous studies (Martinez et al., 2011; Gohel & Singh, 2012). E_a is directly related to the activation enthalpy of denaturation (ΔH) (equation 5) which is another important thermodynamic parameter expressing the total amount of energy required to denature the enzyme. The thermal denaturation of enzymes is followed by a breakage of non-covalent linkages, including hydrophobic interactions, with a related increase in enthalpy of activation (Figure 11). The disruption of the enzyme structure is accompanied by an increase in disorder, randomness or entropy of activation. The positive values of E_a and ΔH shows high enzyme stability. ΔH at 40°C is (18.69 kJ mol^{-1}) as shown in Table 6. The thermal enzyme denaturation also depends on the activation entropy (ΔS), which

Table 5 Kinetic parameters for inactivation of enzyme

T°C	R^2	k^{-1}(min)	$t_{1/2}$(min)	D(min)
30	0.977	0.0028	247.7091	822.8719
40	0.9702	0.0031	224.4061	745.4608
50	0.9929	0.0046	152.0058	504.9522
60	0.9949	0.0057	121.0385	402.0813
70	0.9956	0.0071	98.1002	325.8819

T°C- temperature; k^{-1}- thermal inactivation; $t_{1/2}$- half life; D –Decimal reduction time; R^2 –Coefficient of determination.

Table 6 Thermodynamic parameters of denaturation of protease

E_a (kJ mol^{-1})	T (K)	Δ H (kJ mol^{-1})	Δ G (kJ mol^{-1})	Δ S (kJ mol^{-1})
21.2953	303	18.78	89.01493	-0.23181
	313	18.69	91.78019	-0.2335
	323	18.61	93.75131	-0.23263
	333	18.53	96.1078	-0.23297
	343	18.44	98.47935	-0.23334

E_a – activation energy; T- temperature; ΔH- enthalpy; Δ G- Gibbs free energy; Δ S-entropy

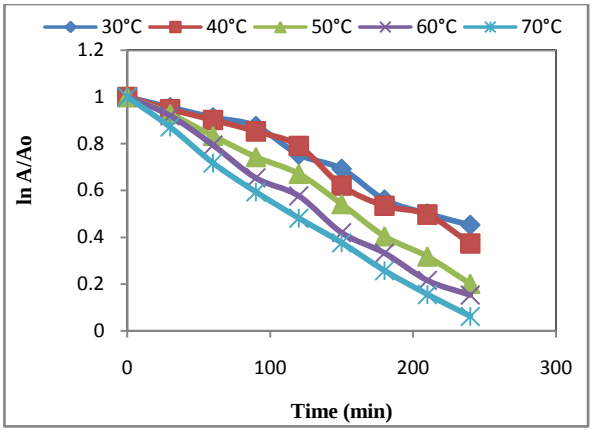

Figure 11 Thermal inactivation of protease at 30, 40, 50, 60 and 70°C up to 240 min

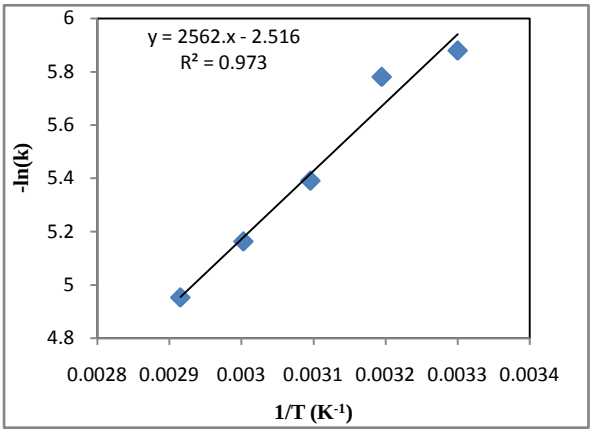

Figure 12 Arrhenius plot of thermal in activation of enzyme

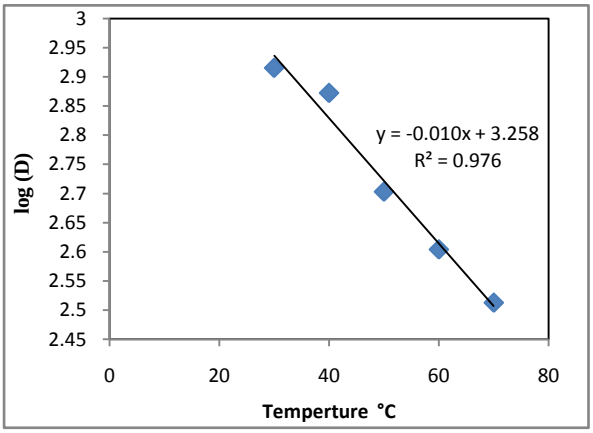

Figure 13 Decimal reduction time (D) variation of enzyme at different temperature

expresses the amount of energy per degree involved in the change from a native to a denatured state. ΔS values lie from -0.23334 to -0.23181kJmol^{-1}. A negative entropy suggests the negligible disorder and thereby reasonable thermostability at high temperature values (30, 40, 50, 60 and 70°C) (Martinez et al., 2011; Gohel & Singh, 2012).

Another important thermodynamic parameter, the Gibbs free energy of denaturation (ΔG), includes both enthalpic and entropic contributions; therefore, it is a more accurate and reliable predicting tool to evaluate enzyme stability. The smaller or negative value of ΔG corresponds to the more spontaneous nature of the process. This means that the enzyme becomes less stable and undergoes denaturation very easily (Mehta et al., 2006). Increase in ΔG corresponds to increase in thermostability (Gohel & Singh, 2012). It was observed that the value of ΔG increased with the temperature. Our results are in line to the previously reported ones, which too report that the ΔG got increased with increase in temperature of protease from *Aspergillus fumigates* (89.2 to 91.4 kJ mol^{-1}) and *Aspergillus awanori* (103.1 to 113.8 kJ mol^{-1}), respectively (Martinez et al., 2011; Melikoglu et al., 2013).

Conclusion

The present study was aimed at the optimization of cultural conditions, kinetics and thermodynamic properties of extracellular protease produced by *H. fuscoatra* MTCC 1409 using biodigested slurry as a substrate. It was found that 25% slurry concentration, 10% inoculum concentration, 40°C temperature and pH 5 are the best conditions to produce maximum protease (531 Ug^{-1}) from poultry dropping based biodigested slurry. Residual activity determined at 30-70°C, at different intervals of time, was used to determine enzyme $t_{1/2}$ (224.4061 at 40°C), and thermodynamic parameters of the irreversible denaturation of enzyme. The enzyme exhibited a ΔH of 18.69 kJ mol^{-1}, ΔS of -2.335 kJ mol^{-1}and ΔG of 91.78 kJ mol^{-1}. These values determine the thermostability of enzyme that could be used for future industrial applications such as in detergent, paper and textile sectors etc. Further studies are required for its implementation on large scale production to reveal the potential application of proteases. These results not only depict value addition of biodigested slurry but also large scale slurry but also large scale slurry management which otherwise is a source of environmental menace.

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Conflict of Interest

Authors would hereby like to declare that there is no conflict of interests that could possibly arise.

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