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Statistical Optimization of Keratinase Production from Marine Fungus

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Abstract
To improve the yield of keratinase from marine fungus *Scopulariopsis brevicaulis*, different medium constituents were optimized using response surface methodology (RSM) based on central composite design (CCD). The strain produced 24.8U/mL and 36.4U/mL of keratinase activity in conventional method of optimization with glucose and soya bean meal as carbon and nitrogen sources. Response surface methodology which was applied to optimize concentrations of glucose, soya bean meal, feather powder and inoculum level, improved the productivity to 225.0U/mL. This value represents 6.18 fold increases in productivity as compared to conventional methods. Optimal parameters of the cultivation process were determined as glucose 1.52g/L, soya bean meal-1.08g/L, feather powder-1.04g/L and inoculum level-10.6%.

Keywords: Keratinase, Marine sponge, Response surface methodology, *Scopulariopsis brevicaulis*, Glucose, soya bean meal.

I. Introduction

Chicken feathers are produced from poultry farms as undegradable waste material. Feathers are composed with keratin. Keratins are highly stable, insoluble fibrous proteins composed of tightly packed α-helix (α-keratin, e.g., hairs) or β-sheet (β-keratin, e.g., feathers) intertwined polypeptide chains which form dense network of intermediate filaments (IF) (Cohlberg, 1993). Keratin is the major constituent of skin, nails, hair, horns and wool. The high number of disulfide bonds in keratin makes it insoluble and indigestible by nature ((FilipelloMarchisio, 2000). Feathers are processed to animal feed material using thermal (Wang and Parsons, 1997) and chemical methods. But the enzymatic hydrolysis is the most convenient method to convert insoluble waste as soluble material without producing air pollution. Biodegradation of keratin containing feathers were achieved by incubating feathers with keratinase producing organisms. Production of Keratinase from various terrestrial organisms was reported previously by Williams *et al*., (1990) and Shankar *et al*., (2014) from *Bacillus*, Noval and Nickerson (1959) and Amany *et al*., (2009) from *Streptomyces*, Orr (1969) and Jitendra Kumar and Kushwaha (2014) from fungi. keratinases are having high specificity and efficiency in bio-degradation of insoluble feathers to animal feed stuff. Keratinases are also useful in cosmetic technology, leather and textile industry, in ungual drug delivery (Adriano Brandelli, 2008). Because of wide applications, keratinases are gaining importance commercially. So, the present research focused to isolate new marine fungus from sponges where as very few organisms were reported previously from marine sources. These marine organisms are having more salt tolerance and abundant keratinase producing ability. To reach the industrial needs it required to improve the yield of enzyme by application of optimization methods. Compared to conventional methods statistical methods are more advantageous as they required less time and simultaneously more than one parameter is optimized at five different levels. The present study was focused to optimize medium constituents for keratinase production by isolating fungus from marine sponges. Statistical methods like Response surface methodology (RSM) was applied to find out the effect of individual component on keratinase production and interaction between different components.

II. Materials and Methods

2.1 Isolation of organism

Fungus strain *Scopulariopsis brevicaulis* (MTCC 11794) isolated from marine sponges collected from Kulasekarapattinam, Tuticori district, Tamil Nadu using soluble keratin medium. Keratin substrate was prepared by method of Wawrzkiewicz *et al*., (1991).

2.2 Production of keratinase

Keratinase from marine fungus was produced from the production medium containing $MgSO_4.H_2O$-0.5; KH_2PO_4-0.1; $FeSO_4.7H_2O$-0.01; $ZnSO_4.7H_2O$-0.005 g/L; Distilled water-500mL and Sea water-500mL; pH adjusted to 7.5 and the plates were incubated up to 5 days at room temperature ($28^0C \pm 2^0C$) (Saber *et al*., 2009).

2.3 Assay of keratinase:

Keratinolytic activity was assayed as follows: 1mL of crude supernant was diluted in 0.2M, pH 7.0 Tris-acetate buffer and incubated with 0.5% of 1mL of soluble keratin solution at 50^{o}C for 30min; reaction was arrested by adding 15% of TCA. After cooling the mixture was centrifuged and absorbance was determined at 280nm against control. One unit (U/mL) of keratinolytic activity was defined as an increase of corrected absorbance of 280 nm (*A*280) (modified method by Gradisar *et al*., 2005) with the control for 0.01 per minute under the conditions described above and calculated by the following equation:

$$U=4\times n\times A280/ (0.01\times T)$$

Where n is the dilution rate; 4 is the final reaction volume (ml); T is the incubation time (min).

2.4 Screening of carbon and nitrogen sources by conventional method

11 carbon and 11 nitrogen sources were screened to identify the best carbon and nitrogen components for highest keratinase production. The following different carbon and nitrogen sources were tested such as glucose, glycerol, sucrose, lactose, sorbitol, mannitol, mannose, saccharose, potato starch, soluble starch, fructose and casein, yeast extract, peptone, tryptone, soya bean meal, urea, glycine, tyrosine, KNO_3, $NaNO_3$, meat extract. 0.2% of sample concentration was incorporated in basal mineral medium with 10% of fresh inoculum and incubated at 28^{O}C, 120 rpm for about 5 days.

III. Statistical optimization

3.1 Response surface method

The optimum concentration and interaction of signal parameters namely glucose, soya bean meal, feather meal and inoculum level were studied by Response surface method. Basal mineral medium described above with variable concentrations of soya bean meal, glucose, feather meal and inoculum level was used for Response surface method studies. Each factor was studied at five different levels -2, -1, 0, +1, +2. A set of 28 experiments were generated and their actual and coded forms are listed in Table 1. A multiple regression analysis was performed on the data obtained.

The following second-order polynomial equation was adopted to study the effects of variables to the response.

$$Y=\beta_o + \beta_1X_1 + \beta_2X_2 + \beta_3X_3 + \beta_4X_4 + \beta_{11}X_{11} + \beta_{22}X_{22} + \beta_{33}X_{33} + \beta_{44}X_{44} + \beta_{12}X_1X_2 + \beta_{13}X_1X_3 + \beta_{14}X_1X_4 + \beta_{23}X_2X_3 + \beta_{24}X_2X_4 + \beta_{34}X_3X_4 \text{ ---------- (1)}$$

Where Y is the response (keratinase yield, U/mL), βo is the intercept term, β_1, β_2, β_3, β_4 the coefficients of linear terms and β_{11}, β_{22}, $\beta_{33,}$ β_{44} are the coefficients of quadratic terms and β_{12}, β_{13}, β_{14}, β_{23}, β_{24}, β_{34} are the coefficients of interaction, X_1, X_2, $X_{3,}$ X_4 represent the factors feather meal, glucose, soya bean meal, and % of inoculum respectively.

IV. Results

4.1 Isolation of keratinase producing organisms

The main interest of the present investigation is to isolate marine organisms having keratinase producing ability using cheap substrate like chicken feathers. Marine sponges were collected from Kulasekarapattinam, Tuticori district, Tamil Nadu. Among all isolates one fungus showing maximum keratinolytic activity (Figure.1) on soluble keratin containing mineral medium was selected for further study and it was identified as *Scopulariopsis brevicaulis* by IMTECH, Chandigarh, India.

4.2 Keratinase production

Keratinase activity was assayed after 5 days of incubation in mineral salt medium utilizing chicken feathers as carbon and nitrogen sources. Without any prior treatment of feathers the present isolate degrading them in 5 days of short incubation period to produce keratinase. Several tons of feathers were produced from poultry industry every year and denaturation by some conventional methods like thermal and chemical treatment leads to air pollutants. Enzymatic and microbial hydrolysis is the effective alternative methods to denature feathers without causing air pollution. 16.06U/mL of keratinase activity was obtained after growing on basal mineral medium.

4.3 Optimization of carbon and nitrogen sources

Among selected 11 carbon and 11 nitrogen sources glucose and soya bean meal were recorded as best sources for keratinase production with 24.8 and 36.4 U/mL of keratinase activity (Figure 2 and 3). Mannose and glycine recorded minimum keratinse production.

4.4 Statistical optimization

The fitted second order response surface model as specified by Eq. (1) for keratinase activity (U/mL) in coded process variables is:

Y= 224.87 + 0.6954 X1 + 1.4571 X2 + 7.0304 X3 + 3.1038 X4 -8.8230 X12 -9.3918 X22 -15.4730 X32 - 8.6318 X42 + 5.7956 X1* X2 + 6.1769 X1*X3 + 3.7056 X1*X4 -4.6569 X2*X3 -3.3256 X2*X4 - 1.8069X3*X4 ---------- (1)

ANOVA results of the model shown in Table 2 & 3. The goodness of the fit of the model verified by the determination coefficient R^2 value. In this study, the R^2 value for the keratinase production was 0.946. The value of the adjusted determination coefficient (Adj R^2= 0.888) advocates a high significance of the model. The Predicted R^2 of 0.7206 is in reasonable

agreement with the adjusted R^2 of 0.8889. At the same time, a relatively lower value of the coefficient of variation (CV = 3.6%) indicates a better precision and reliability of the experiments carried out (Myers and Montgomery, 1995; Khuri and Cornell, 1987).

The surface (3D) and contour (2D) plots based on Eq. (1) were prepared using STATISTICA 7.0 software. The surface plot (Figure 4a-4f) shows the behavioural change with respect to simultaneous change in two variables.

The behaviour of keratinase production with respect to change in glucose and soya bean meal concentrations at specific hold values is shown in Figure 4a. From the figure it was observed that the contour plot is centered between soya bean meal and glucose indicating that the interaction between these two parameters is significant. It was observed that glucose at 1.48-1.6g/L(Figure 4a-4c) and soya bean meal at 0.98-1.18g/L (Figure 4a, 4d & 4e) concentrations were effective for enzyme production.

Feather meal is the main source to produce keratinase from different organisms. From the figure 4b, 4d and 4f it was noticed the interaction behaviour of feather powder with other variables. It was observed that concentration of feather powder slightly depends on the soya bean meal concentration (Figure 4d). It was observed that feather powder concentration around 0.95-1.1 g/L is optimum for keratinase production (Figure 4b, 4d & 4f). It was noticed that the concentration of inoculum level around 11-9.7% is optimum for keratinase production (Figure 4c, 4e & 4f). From central composite design the optimum concentration for glucose, soya bean meal, feather powder and inoculum level were observed to be 1.52g/L, 1.08g/L, 1.04g/L and 10.6% respectively. The predicted enzyme production was 224.87U/mL. While conducting the experiments at the predicted optimum conditions, the keratinase production obtained was 225.00U/mL.

V. Discussion

Keratinases are the class of proteolytic enzymes. These enzymes are finding their applications in several industries. Keratinases utilizes keratin as substrate, which is the most important waste material produced high amount from slaughter house and poultry farms in the form of insoluble, undegradable feathers. In the present study keratinase was produced from marine fungus *Scopulariopsis brevicaulis*. Keratinase production from *Scopulariopsis brevicaulis* was previously reported by Anbu, Eman and Neveen and Malviya and maximum enzyme production possible only after 5 weeks and they were isolated same organism from poultry farm soil and Egyptian black soil. As compared to previous reports maximum amount of enzyme production was possible with short incubation period. RSM was successfully applied to the production of keratinase by Zauari *et al*., 2010. The present study showed that the maximum keratinase enzyme production was 225.0U/mL. Four factors glucose, soya bean meal, feather powder, inoculum level were optimized in this present study. The factors like glucose, soy flour and incubation time were employed for RSM optimization by Ekta Tiwary and Gupta 2010. The factors used by Zauari were feather meal, soy peptone, sodium chloride, potassium chloride and potassium dihydrogen phosphate and the factors like sucrose, yeast extract and feather keratin were used by Xian *et al*., 2010. Twenty eight run experimental set up was used in RSM in this present study for the production of enzyme keratinase. Twenty run experimental setup by Tiwary and Gupta and seven experimental setups for maximizing the production of keratinase using RSM were demonstrated by Xian *et al*. Similar work for keratinase production using response surface methodology was reported by Zauari *et al*., 2010, Xian *et al*., 2010 and Ekta Tiwary and Gupta, 2010. The medium components play an important role in keratinase production by fungus. Therefore designing an appropriate fermentation medium is of critical importance in optimizing the product yield.

VI. Conclusion

The statistical methods used in the present study enabled us to optimize the keratinase production medium, giving a 6.18-fold increase compared with the unoptimized medium. Thus statistical studies may prove useful for optimization of keratinases production by other micro-organisms too, thereby making their industrial application more promising.

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

Variables	Actual	coded	actual	Coded	actual	coded	Actual	coded	actual	Coded
F.M (% w/v)	0.5	-2	0.75	-1	1.0	0	1.25	+1	1.5	+2
Glucose (% w/v)	0.5	-2	0.8	-1	1.0	0	1.2	+1	1.5	+2
S.B.M (% w/v)	0.5	-2	0.8	-1	1.2	0	1.5	+1	1.8	+2
Inoculum level (% v/v)	5	-2	8	-1	10	0	12	+1	15	+2

F.M- Feather meal, S.B.M- Soya bean meal.

Table:2 Central composite design (CCD) of factors in coded value for optimization of process variables

Trails	**Type**	**F**	**G**	**S**	**I**	**Observed Keratinase activity(U/ml)**	**Predicted Keratinase activity(U/ml)**	**Residual Keratinase activity(U/ml)**
1	Factorial	-1	-1	-1	-1	179.3900	176.1575	3.23250
2	Factorial	-1	-1	-1	1	180.9100	185.2188	-4.30875
3	Factorial	-1	-1	1	-1	190.0300	190.7921	-0.76208
4	Factorial	-1	-1	1	1	191.5500	192.6258	-1.07583
5	Factorial	-1	1	-1	-1	186.9900	183.4454	3.54458
6	Factorial	-1	1	-1	1	180.9200	179.2042	1.71583
7	Factorial	-1	1	1	-1	179.3900	179.4525	-0.06250
8	Factorial	-1	1	1	1	159.6200	167.9838	-8.36375
9	Factorial	1	-1	-1	-1	152.0200	146.1921	5.82792
10	Factorial	1	-1	-1	1	173.3100	170.0758	3.23417
11	Factorial	1	-1	1	-1	186.9900	185.5342	1.45583
12	Factorial	1	-1	1	1	196.1100	202.1904	-6.08042
13	Factorial	1	1	-1	-1	180.9100	176.6625	4.24750
14	Factorial	1	1	-1	1	185.4700	187.2438	-1.77375
15	Factorial	1	1	1	-1	199.1500	197.3771	1.77292
16	Factorial	1	1	1	1	200.6700	200.7308	-0.06083
17	Axial	-2	0	0	0	191.5500	188.1921	3.35792
18	Axial	2	0	0	0	186.9800	190.9738	-3.99375
19	Axial	0	-2	0	0	183.9500	184.3938	-0.44375
20	Axial	0	2	0	0	190.0300	190.2221	-0.19208
21	Axial	0	0	-2	0	141.3800	148.9221	-7.54208
22	Axial	0	0	2	0	183.9500	177.0438	6.90625
23	Axial	0	0	0	-2	174.8300	184.1404	-9.31042
24	Axial	0	0	0	2	205.2300	196.5554	8.67458
25	Center	0	0	0	0	221.9500	224.8750	-2.92500
26	Center	0	0	0	0	232.1200	224.8750	7.24500
27	Center	0	0	0	0	220.4300	224.8750	-4.44500
28	Center	0	0	0	0	225.0000	224.8750	0.12500

Table: 3 Anova values for keratinase production by RSM

F value	16.43
P>F	0.000
R2	0.946
Adjusted R2	0.888
Predicted R2	0.72
Correlation variance	3.6

Fig: 1: *Scopulariopsis brevicaulis* showing keratinolytic activity on soluble keratin medium

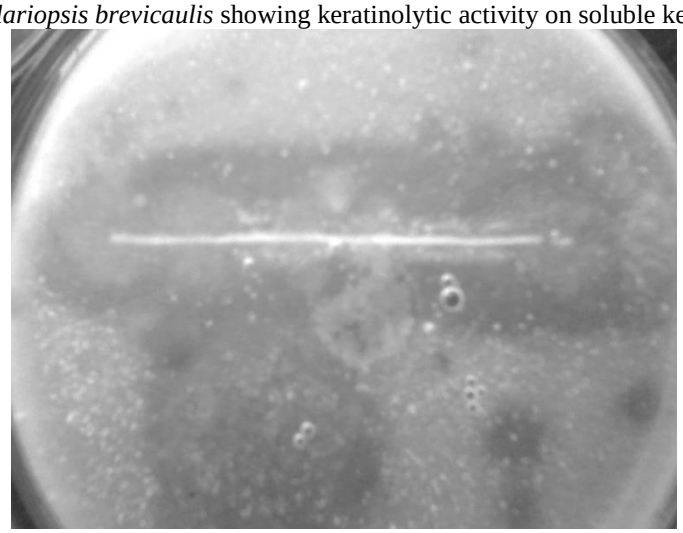


Fig: 2: Effect of carbon source on keratinase production from *S.brevicaulis*

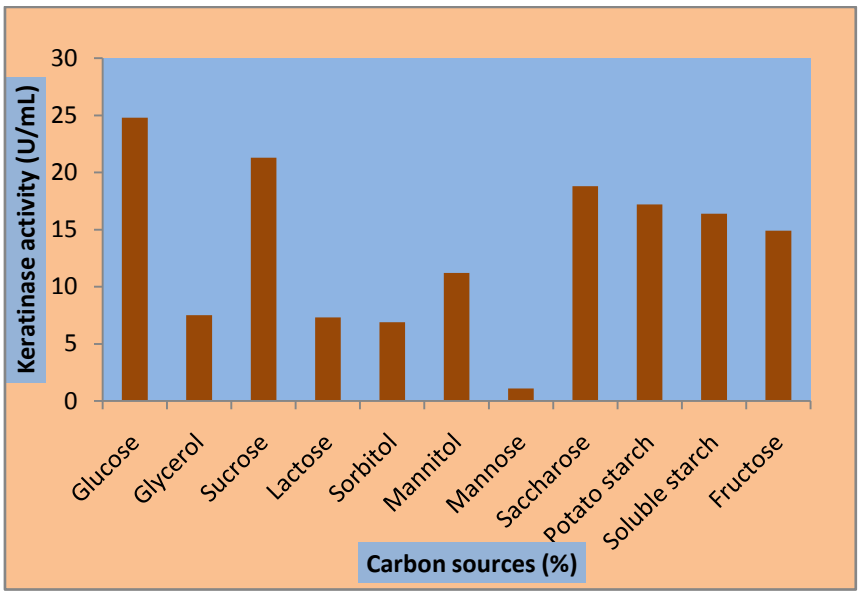


Fig: 3: Effect of nitrogen source on keratinase production from *S. brevicaulis*

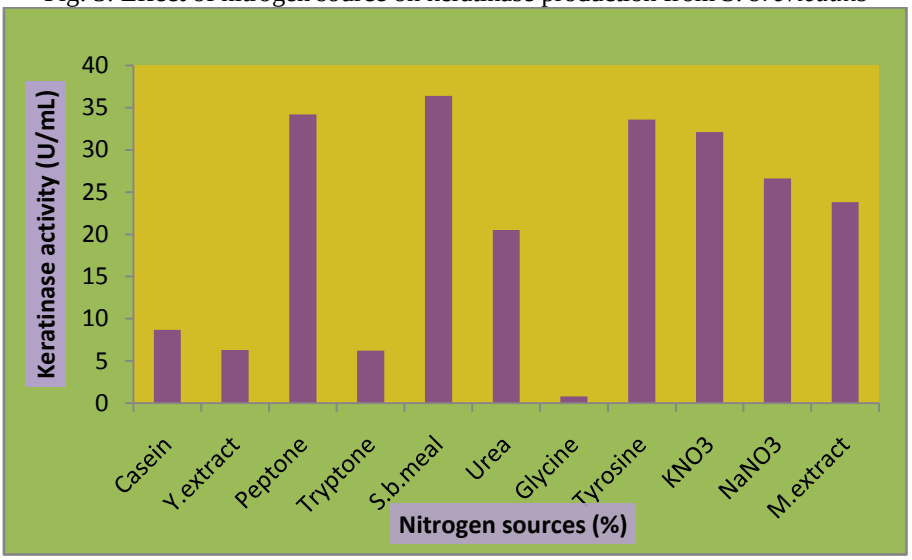


Fig: 4a Contour plot showing the effect of Glucose and S.B.M on keratinase production

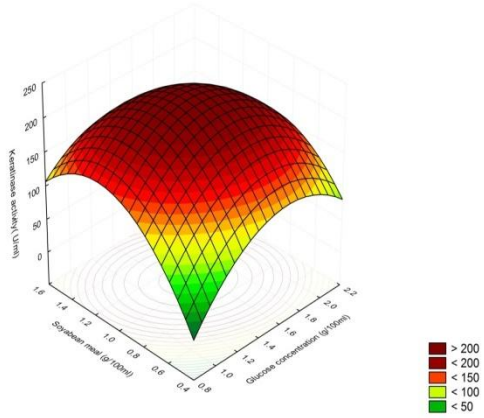


Fig: 4bContour plot showing the effect of Glucose and feather meal on keratinase production

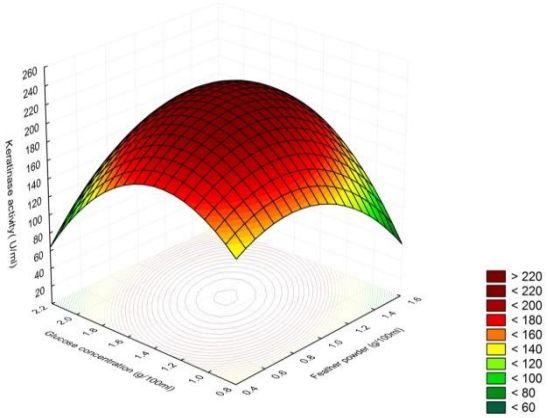


Fig: 4d Contour plot showing the effect of Inoculums and glucose on keratinase production

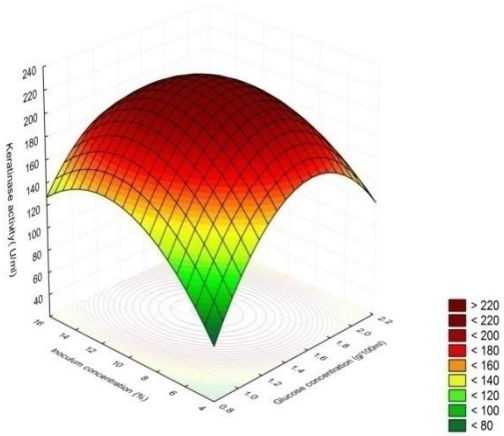


Fig: 4c Contour plot showing the effect of S.B.M and feather powder on keratinase production

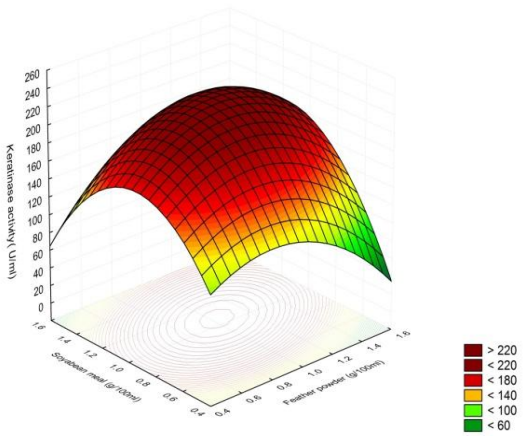


Fig: 4e Contour plot showing the effect of Inoculums and S.B.M on keratinase production

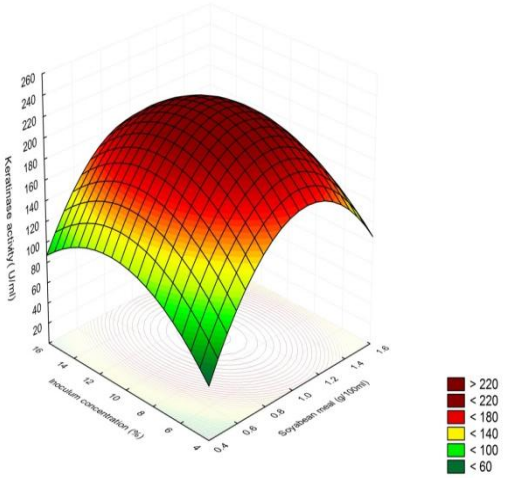
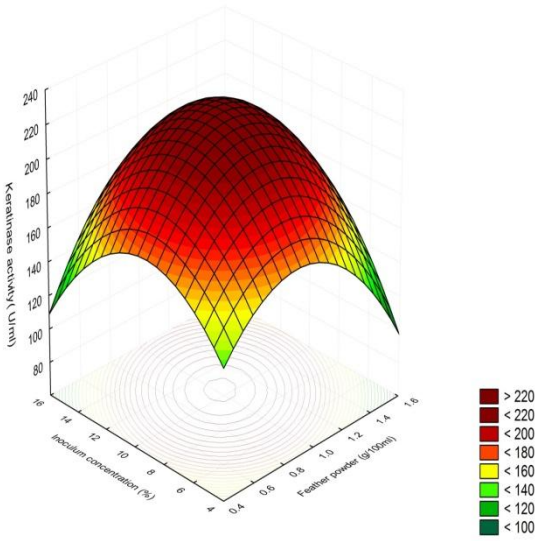


Fig: 4f Contour plot showing the effect of inoculums and feather powder o keratinase production



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