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Optimization of process parameters for keratinase enzyme production using statistical experimental design

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Abstract: ***Keratinases are exciting proteolytic enzymes that display the capability to degrade the insoluble protein keratin. Keratinases display a great diversity in their biochemical and biophysical properties. The effect of different production parameters such as initial pH, temperature, incubation period, inoculum size and substrate concentration on keratinase production by the bacterial strain Bacillus subtilis NCIM 2724 was studied. The enzyme production was assayed in submerged fermentation (SmF) condition. Complete feather degradation was achieved at pH 8, inoculum size 1.5% w/v, agitation speed 200 rpm and incubation temperature 40^0C , incubation period of 48 h which represents the late log phase. Submerged fermentation was carried out with 48 h incubation period, temperature 40^0C, initial pH 8.0, substrate concentration (1.5% w/v) and inoculum size (4% v/v) and were chosen based on maximum keratinase enzyme activity. The optimum conditions for a maximum keratinase activity of 120.08 IU/ml with B. subtilis using Chicken feather powder as substrate and were found to be incubation period of 46.846 h, temperature of 41.681^0C, pH of 8.1681, substrate concentration of (1.5120 % w/v) and inoculum size of (3.9898 % v/v).***

Keyword: **Feather keratins, Degradation, Keratinase, Keratinolytic activity; optimization, physico chemical parameters, response surface methodology.**

I. INTRODUCTION

Feathers are the major by-product of chicken poultry and it is gradually increasing day by day. Specifically around 8.5 billion tons of chicken feathers are produced annually world wide as a waste from the production unit of chicken meat and the India's contribution alone is 350 million tons [1]. Accumulation of such huge volume of feathers which is recalcitrant in nature , leads to environmental pollution and feather protein wastage [2,3]. The feather is rich resource because it contains over 90% (w/v) keratins [4]. Now a days the most commonly used industrial enzymes belong to the hydrolase group, which exploits several natural substrates [5]. However keratin can be degraded by keratinases produced by some species of saprophytic and parasitic fungi [6], Actinomyces [7], and bacteria , especially of the genus *Bacillus* [8].

Industrial fermentation is moving away from traditional and largely empirical operation towards knowledge based and better controlled process [9]. A number of optimization techniques can be used for this purpose. Statistical approaches offer ideal ways for process optimization studies in biotechnology [10]. Time consuming, requirement of more experimental data sets [11] and missing the interactions among parameters are the obstacles in predicting the accurate results when the conventional optimization procedures like 'one-factor at a time' were applied [12]. Response surface method (RSM) is one of the popularly used optimization procedures, mainly developed based on full factorial central composite design (CCD) [13]. RSM is a collection of mathematical and statistical techniques that are useful for modeling and analysis in applications where a response of interest is influenced by several variables and the objective is to optimize this response. The first step in RSM is finding a suitable approximation for the true relationship between the response and independent variables. Usually a low order polynomial in some region of the independent variables is employed for modeling. If the response is well modeled by a linear function of the independent variables , then the approximating function is the first order . If there is curvature in the system, then a polynomial of higher degree must be used such as the second- order model [14]. RSM helps identify the effective factors, study interactions, select optimum conditions and quantify the relationships between one or more measured responses [12]. CCD's are designed to estimate the coefficients of a quadratic model [14]. The central composite design was originally introduced by Box and Wilson (1951). In this study keratinase production from *Bacillus subtilis NCIM-2724* as a result of the interaction between 5 variables such as incubation period, temperature, initial pH, substrate concentration and inoculum size which had played a significant role in enhancing the production of keratinase was optimized with CCD of response surface methodology.

II. MATERIALS AND METHODS

A. Preparation Of Substrate

Chicken feathers collected from nearby poultry farm were used as substrate. Feathers were washed several times with distilled water and subsequently dried initially in sunlight followed by drying in hot air oven at 50°C. The feathers were pre-treated in chloroform: methanol

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(1:1) solution for 48 hrs followed by drying at approx 40°C and storage at 4°C [15].

B. Organism Medium And Growth Conditions

Keratinase production was carried out in the previously optimized medium of mineral constituents comprising of peptone (0.53939%w/v), NaCl (0.09485 % w/v), KCl (0.09485 %w/v) and K_2HPO_4(0.13384 %w/v). 100 ml of the above mentioned medium was poured in to the 250 ml of Erlenmeyer flask and inoculated with 4(%v/v) of *B. subtilis NCIM 2724* and incubated at 32^{0}C in an orbital shaker set at 200 rpm for the experimental time. The cells from the culture were separated by centrifugation and the bacteria were removed by filtration. Around 10 ml of culture filtrate was taken in a sterile eppendorf tubes and were centrifuged at 6000 rpm for 5 min in a cold room. The supernatant was transferred carefully in to another test tube and stored at 4^{0}C for Keratinase enzyme assay [15].

C. Central Composite Design (CCD)

The experimental design and statistical analysis were performed according to the response surface analysis method using Design-Expert 8.0.6.1 (Stat-Ease, 2010) version software. Analysis of variance (ANOVA) and response surface plots were generated using Design- Expert 8.0.6.1. CCD for five variables and three levels each with four concentric point combinations was used to find the optimized process variables for the keratinase enzyme production (IU/ml).

The overall second order polynomial mathematical relationship of the response Y (Keratinase, IU/ml) and the five variables affecting the karatinase activity is represented by quadratic Eq. (1).

The experimental design for the variables, i.e. Incubation time 16 - 80 h, temperature 20- 60^{0}C, pH 6 - 10 , substrate concentration 0.5- 2.5% w/v and Inoculum size 2 - 6% w/v were taken for measuring the enzyme activity. The design was applied for selection range of each variable (minimum and maximum), total of 50 experiments were designed by the model and performed.

The design consist of 2^5 CCD factorial points having eight replicates at the central point and ten axial points (α). Optimized values of five independent variables for maximum activities were determined using a numerical optimization package of Design-Expert 8.0.6.1.

D. Keratinase Assay

Keratinolytic activity was measured using a modified protocol of [16] with soluble keratin as a substrate. One ml of crude enzyme solution is diluted with 3 ml of phosphate buffer (0.05 M of pH 7.0) and 1 ml of keratin solution (0.5%w/v) was added and incubated at 50^{0}C for 10 min. After the incubation 2 ml of 0.4 M TCA was added to arrest the reaction. Then it was centrifuged at 1450 g for 20 min and filtered. 2.5cc of 0.5N $NaHCO_3$ solution and 0.5 cc of diluted folin's reagent were added to 1 ml of the filtrate obtained above. Then it is incubated at 37^{0}C for 30 min until blue color developed. Then it was centrifuged at 1450g for 10 min and the absorbance of the supernantant liquor was read at 660 nm. A tyrosine standard curve was made for quantification, and was generated using solutions of 50–500 µg/ml of tyrosine. The enzyme activity is said to be one IU/ml when the amount of enzyme required to liberate 1 micro mol tyrosine in 1 min. The unit of keratinase activity is defined as µg of tyrosine liberated per min per ml of enzyme under the experimental conditions used [22].

E. Results

Screening of most important variables and their optimization was attempted to improve the enzymatic yield under SmF on the pretreated powdered chicken feather was done. The experimental CCD for the five process variables / parameters was studied for measuring the keratinase production.

The design was applied for selection of each parameter (maximum and minimum) as shown in Table 1. Total 50 experiments were designed and performed (Table 2).

The experimental results associated with processing set of each independent variable are listed in Table 1. To study the combined effects of these factors/ variables, experiments were conducted at different combinations

Table: 1 CCD of actual and coded levels of variables for the optimization of variables [23]

Variables	Code	Levels (% w/v)				
		-2.378	-1	0	+1	+2.378
Incubation period (h)	A	16	32	48	64	80
Temperature (°C)	B	20	30	40	50	60
Initial pH	C	6	7	8	9	10
Substrate concentration (%w/v)	D	0.5	1.0	1.5	2.0	2.5
Inoculum size (%v/v)	E	2	3	4	5	6

$$Y = +119.95 - 1.55A + 1.60B + 2.76C + 0.45D - 0.18E - 3.16AB + 3.91AC - 0.37AD - 2.31AE + 0.92BC + 1.35BD + 0.54BE + 0.50CD - 1.91CE + 1.28DE - 10.64A^2 - 6.28B^2 - 7.33C^2 - 9.69D^2 - 8.12E^2 \quad \ldots (1)$$

Where Y = Predicted response (keratinase, IU/ml), A, B, C, D and E are the coded values of independent variables such as Incubation period, temperature, initial pH, substrate concentration and inoculum size respectively. The regression equation was used to calculate the predicted responses and are given in Table 2. Comparison of the predicted values with the experimentally obtained actual values indicated that these data are in reasonable agreement (Fig. 11).

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TABLE: 2 Experimental conditions (coded values) and observed response values of 2^5 central composite design for Keratinase enzyme production

S. No	Incubation period (h)	Temperature (0C)	pH	substrate conc (%w/v)	Inoculum size (%v/v)	Exp Keratinase activity (IU/ml)	Predicted keratinase activity (IU/ml)
1.	0	0	0	0	-2.37841	76.82	74.4494
2.	0	0	0	0	0	120.08	119.9731
3.	1	1	-1	1	1	69.08	69.36994
4.	-1	-1	1	-1	1	71.02	71.04458
5.	0	0	0	0	0	120.08	119.9731
6.	0	2.378414	0	0	0	90.97	88.20587
7.	0	0	0	-2.37841	0	60.665	64.06717
8.	0	0	0	0	0	120.08	119.9731
9.	-1	1	1	1	-1	82.972	84.59521
10.	-1	-1	-1	1	-1	68.29	70.93407
11.	0	0	-2.37841	0	0	68.9	71.90235
12.	1	1	-1	-1	-1	64.09	67.56706
13.	0	0	0	0	0	120.08	119.9731
14.	-2.37841	0	0	0	0	61.179	63.46526
15.	2.378414	0	0	0	0	60	56.08681
16.	1	1	1	1	-1	82.95	86.86532
17.	1	-1	1	1	1	79.23	79.23195
18.	1	1	-1	1	-1	66.89	66.87007
19.	-1	1	-1	-1	1	88.97	86.05257
20.	0	0	0	0	0	120.08	119.9731
21.	0	0	2.37841 4	0	0	89.67	85.04073
22.	1	-1	-1	-1	-1	78.33	76.31556
23.	1	-1	1	1	-1	87.45	86.51957
24.	0	0	0	2.378414	0	71.23	66.20091
25.	0	0	0	0	0	120.08	119.9731
26.	-1	1	-1	1	1	87.94	91.95632
27.	-1	1	1	-1	-1	79.9	81.84796
28.	1	-1	1	-1	-1	87.67	90.62907
29.	-1	-1	-1	-1	1	77.89	79.99182
30.	-1	1	-1	-1	-1	77.98	79.4677
31.	1	-1	-1	1	-1	67.9	70.22294
32.	1	1	-1	-1	1	67.09	64.92731
33.	0	0	0	0	0	120.08	119.9731
34.	-1	-1	-1	1	1	85.473	80.49994
35.	-1	1	-1	1	-1	84.32	80.23182
36.	-1	-1	1	1	-1	67.9	71.59883
37.	-1	-1	1	1	1	73.89	73.53583
38.	-1	-1	-1	-1	-1	79.76	75.56557
39.	0	0	0	0	0	120.08	119.9731
40.	1	1	1	-1	-1	87.45	85.5792
41.	-1	1	1	-1	1	83	80.80395
42.	1	1	1	-1	1	73.469	75.31057
43.	0	0	0	0	2.378414	72.87	73.61368
44.	1	-1	-1	1	1	67.98	70.56419
45.	1	-1	-1	-1	1	74.545	71.51719
46.	-1	1	1	1	1	87.63	88.69083
47.	1	-1	1	-1	1	76.43	78.20182
48.	1	1	1	1	1	78.9	81.73632
49.	0	-2.37841	0	0	0	79.45	80.58721
50.	-1	-1	1	-1	-1	76.9	74.24721

TABLE: 3 Analysis of Variance (ANOVA) for Response Surface Quadratic Model

Analysis of variance table [Partial sum of squares - Type III]

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F
Model	15476.5	20	773.82	70.4	< 0.0001
Incubation period (A)	104.21	1	104.21	9.48	0.0045
Temperature (B)	111.11	1	111.11	10.11	0.0035
pH (C)	330.43	1	330.43	30.06	< 0.0001
substrate concentration(D)	8.72	1	8.72	0.79	0.3805
Inoculum size (E)	1.34	1	1.34	0.12	0.7298
AB	320.08	1	320.08	29.12	< 0.0001
AC	488.71	1	488.71	44.46	< 0.0001
AD	4.27	1	4.27	0.39	0.538
AE	170.19	1	170.19	15.48	0.0005
BC	27.36	1	27.36	2.49	0.1255
BD	58.23	1	58.23	5.3	0.0287
BE	9.32	1	9.32	0.85	0.3647
CD	7.87	1	7.87	0.72	0.4045
CE	116.4	1	116.4	10.59	0.0029
DE	52.83	1	52.83	4.81	0.0365
A^2	6286.63	1	6286.63	571.97	< 0.0001
B^2	2194.36	1	2194.36	199.65	< 0.0001
C^2	2986.82	1	2986.82	271.75	< 0.0001
D^2	5216.83	1	5216.83	474.64	< 0.0001
E^2	3660.58	1	3660.58	333.05	< 0.0001
Residual	318.75	29	10.99		
Lack of Fit	318.75	22	14.49		
Pure Error	0	7	0		
Cor Total	15795.2	49			

Std. Dev.	3.32	R-Squared	0.9798
Mean	83.51	Adj R-Squared	0.9659
C.V. %	3.97	Pred R-Squared	0.9211
PRESS	1246.44	Adeq Precision	9.721

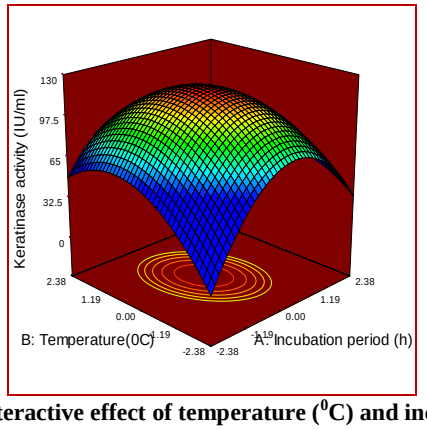

Fig. 1 Interactive effect of temperature (^{0}C) and incubation period (h) on keratinase activity

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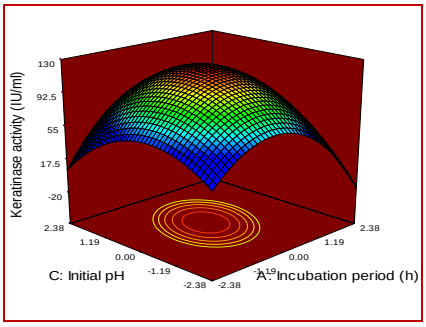

Fig. 2 Interactive effect of initial pH and incubation period (h) on keratinase activity

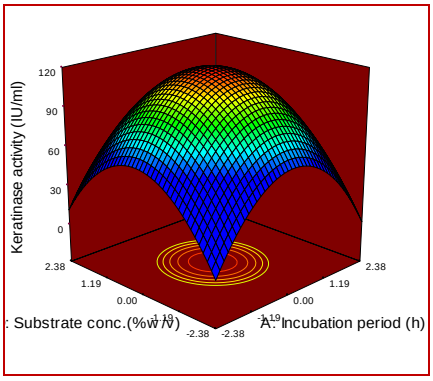

Fig. 3 Interactive effect of substrate concentration (%w/v) and incubation period (h) on keratinase activity

TABLE: 4 Regression analysis and corresponding values of second order polynomial model for the optimization of keratinase production using *B.subtilis* with CFP

Coefficient		Standard	95% CI	95% CI		
Factor	Estimate	df	Error	Low	High	VIF
Intercept	119.95	1	1.16	117.57	122.33	
Incubation period (A)	-1.55	1	0.5	-2.58	-0.52	1
Temperature (B)	1.6	1	0.5	0.57	2.63	1
pH(C)	2.76	1	0.5	1.73	3.79	1
substrate concentration(D)	0.45	1	0.5	-0.58	1.48	1
Inoculum size(E)	-0.18	1	0.5	-1.21	0.85	1
AB	-3.16	1	0.59	-4.36	-1.96	1
AC	3.91	1	0.59	2.71	5.11	1
AD	-0.37	1	0.59	-1.56	0.83	1
AE	-2.31	1	0.59	-3.5	-1.11	1
BC	0.92	1	0.59	-0.27	2.12	1
BD	1.35	1	0.59	0.15	2.55	1
BE	0.54	1	0.59	-0.66	1.74	1
CD	0.5	1	0.59	-0.7	1.69	1
CE	-1.91	1	0.59	-3.11	-0.71	1
DE	1.28	1	0.59	0.086	2.48	1
A^2	-10.64	1	0.44	-11.55	-9.73	1.05
B^2	-6.28	1	0.44	-7.19	-5.37	1.05
C^2	-7.33	1	0.44	-8.24	-6.42	1.05
D^2	-9.69	1	0.44	-10.6	-8.78	1.05
E^2	-8.12	1	0.44	-9.03	-7.21	1.05

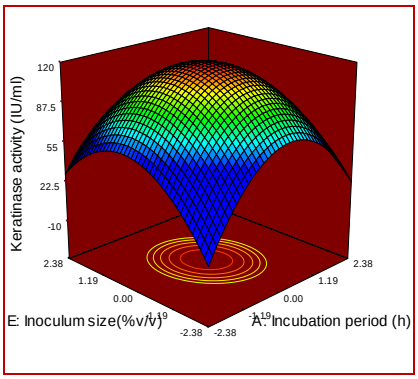

Fig.4 Interactive effect of inoculum size (%v/v) and incubation period (h) on keratinase activity

Table 5 Response optimization

Optimized coded values		Optimized values	uncoded
A	= -0.0720732	46.846 h	
B	= 0.168171	41.6817^0C	
C	= 0.168171	8.168171	
D	= 0.0240244	1.5120 %w/v	
E	= -0.0101347	3.9898%v/v	

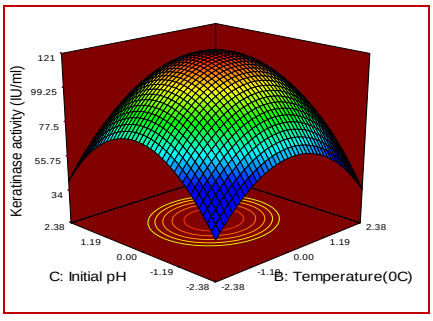

Fig. 5 Interactive effect of initial pH and temperature °C on keratinase activity

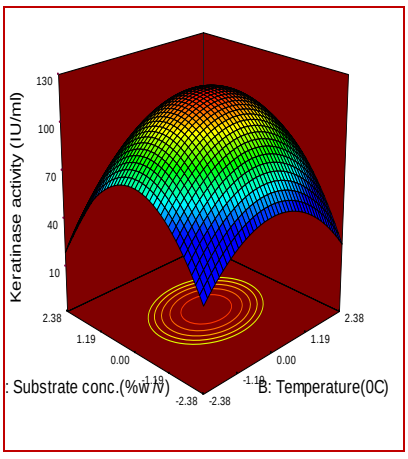

Fig. 6 Interactive effect of substrate concentration (%w/v) and temperature °C on keratinase activity

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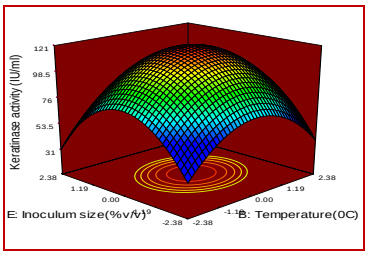

Fig.7 Interactive effect of inoculum size (%v/v) and temperature °C on keratinase activity

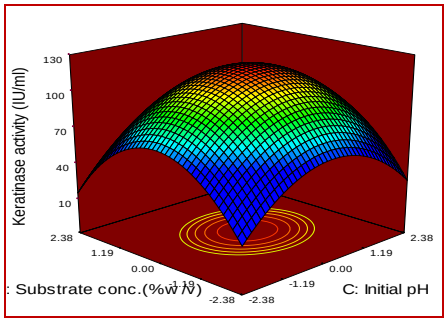

Fig. 8 Interactive effect of substrate concentration (%w/v) and pH on keratinase activity

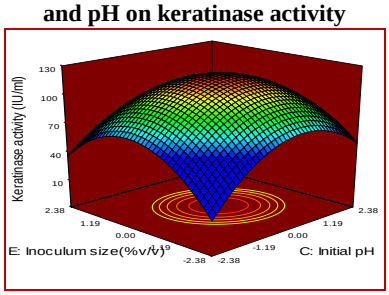

Fig. 9 Interactive effect of Inoculum size (%v/v) and pH on keratinase activity

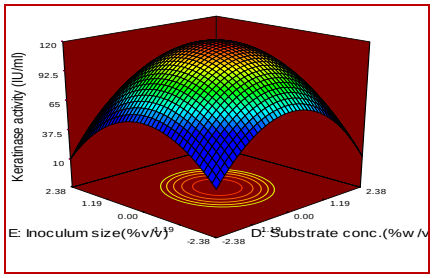

Fig.10 Interactive effect of Inoculum size (%v/v) and substrate concentration (%w/v) on keratinase activity

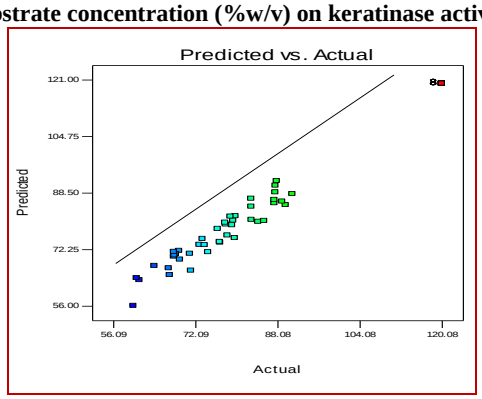

Fig .11 Predicted Vs. Actual (Experimental) Value

III. RESULTS AND DISCUSSION

Statistical testing of the model was done by means of Fisher's statistical test for ANOVA using Design-expert software and the obtained results are presented in Table 3. Generally the 'F' value with a low probability 'P' value indicates high significance of the regression model. The quadratic regression model is significant with a computed F value of 148.27 implies the model is significant (P<F lower than 0.05). The fitness and adequacy of the model was judged by the coefficient of determination (R^2). The R^2 which can be defined as the ratio of the explained variation to the total variation was a measure of the degree of fit. The closer the R^2 value to unity, the better the empirical model fits the actual data. The value of determination of coefficient R^2 is 0.9798, which indicated that model could explain 97.98% of variability and unable to explain only 2.02 % of the total variation. The closer the value of R^2 to 1 indicate the better correlation between the observed and predicted values suggesting a good fit for SmF. The adjusted R^2 was a corrected value for R^2 after elimination of the unnecessary model terms. If many non-significant terms have been included in the model, the adjusted R^2 would be remarkably smaller than the R^2. The adjusted R2 was 0.9659, which is more suitable for comparing models with different numbers of independent variables. The coefficient of variation (CV) is a measure of residual variation of the data relative to the size of the mean; the small values of CV give better reproducibility. A lower value for the CV 3.97% clearly indicate high degree of precision and higher reliability of the experimental values. The significance of individual variables can be evaluated from their P values, the more significant terms having a lower P value. The P values are used to check the significance of each coefficient which also indicates the interaction strength between each independent variable. Table. 3 also gives the P values of each of the variables and their quadratic and interaction terms. Values of "Prob>F" less than 0.05 indicate model terms are significant. In this case A, C, AB, AD, BC, BD, BE, CD, CE, DE, A^2, B^2, C^2 D^2 and E^2 are significant model terms. Values greater than 0.10 indicate the model terms are not significant (Design-Expert® Software. 2010 Version 8 user's guide). Besides the relationship between the actual experimental values and predicted values (Fig. 1) showed that plotted points cluster around the diagonal line, indicating good fitness of the model. Response surface curves were plotted to understand the interaction of variables and for identifying the optimal levels of each parameter for attaining maximum enzyme yield. The response surfaces can be used to predict the optimum range of different variables and the major interactions between the tested variables can be identified from the circular

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or elliptical nature of contours. The highest enzymatic activity 120.08 IU/ml was well agreed with predicted enzymatic activity of 119.97 IU/ml.

The interaction effects of variables on keratinase production were studied by plotting the three dimensional response surfaces with the vertical axis representing enzyme activity (response) and two horizontal axis representing the coded levels of two independent variables, while keeping other variables at their central level (0). The results are shown in Fig 1.0 to Fig 11.

The effect of incubation period on keratinase production in submerged fermentation and the results are shown in Fig.1, Fig.2, Fig.3 and Fig.4. As incubation period was increased from 16 hours to 46.846 hours, the keratinase activity was found to increase and the maximum activity of the enzyme was found at a incubation period of 46.84 hours. For further increase in the incubation period the keratinase activity was found to decrease. Furthermore in spore-forming bacteria secretion of proteinase was often associated with the late logarithmic growth phase or the beginning of sporulation which undoubtedly occurred after depletion of easily available nutrients [18]. Hence 46.84 hours of incubation period was found to be the optimum incubation period and was used for further studies.

Fig. 5, Fig.6 and Fig.7 show the effect of temperature on keratinase activity. As temperature was increased from 20°C to 41.6817 °C, the keratinase activity was found to increase and the maximum activity of the enzyme was found at a temperature of 41.6817°C. The decrease in keratinase activity may be due to denaturation of enzymes at high temperature, growth reduction and enzyme inactivation or suppression of cell viability [20]. Hence the temperature of 41.6817 °C was found to be the optimum temperature and was used for further studies.

The effect of pH on keratinase activity and the results are shown in Fig.2, Fig.5, Fig.8 and Fig.9. As pH was increased from 6.0 to 8.1681, the keratinase activity was found to increase and a maximum activity of the enzyme was found at a pH of 8.1681. Further increase in pH decreases the keratinase activity. This may be due to the preference of bacterial cultures for alkaline medium. Because the metabolic activities of microbes are very much responding to pH change. Previous studies shows that for *B. subtilis*, the highest enzyme production has been reported over a range of pH of 7 to 9 [21]. Hence pH of 8.1681 was found to be the optimum pH and was used for further studies.

The effect of substrate concentration on keratinase activity and the results are shown in Fig.3, Fig.6, Fig.8 and Fig.10. As substrate concentration was increased from 1 to 1.5120 (% w/v) , the keratinase activity was found to increase and a maximum activity of the enzyme was found at 1.5120 (% w/v). Further increase in substrate concentration decreases the keratinase activity. In general many previous studies showed that complete degrading of chicken feathers with various keratinolytic bacteria was only achieved with lower substrate concentration [19]. Hence 1.5120 (% w/v) was found to be the optimum substrate concentration and was used for further studies.

The same trend was obtained for the study of the effect of inoculum size on keratinase activity and the results are shown in Fig.4, Fig.7, Fig. 9 and Fig.10. As inoculum size was increased from 2 to 3.9898 (% v/v), the keratinase activity was found to increase and a maximum activity of the enzyme was found at 3.9898 (%w/v). Further increase in substrate concentration decreases the keratinase activity. An inoculum concentrations higher than the optimum value may produce a high amount of biomass which rapidly depletes the nutrients necessary for growth and product synthesis. On the other hand, lower inoculum levels may give insufficient biomass and allow the growth of undesirable organisms in the production medium [20]. Hence 3.9898 (% v/v) was found to be the optimum inoculum size and was used for further studies.

Optimum Conditions

The optimum conditions for the independent variables as predicted by MINITAB 16 were incubation period 46.846 h, temperature 41.6817^{0}C, pH 8.1681, substrate concentration 1.5120 (%w/v) and inoculum size 3.9898 (% v/v).

IV. CONCLUSION

The process parameters applied in this study demonstrated a good performance. The CCD, regression analysis and response surface method were effective in identifying the optimum condition of keratinase production. Important information was obtained through the RSM with a minimum number of experiments, can effectively optimize the keratinase production. The maximum keratinase production of 120.08 IU/ml was obtained at the optimum process conditions such as incubation period 46.846 h, 8.1681 pH, amount of substrate source 1.5120 (%w/v). These predicted values for optimum process conditions were in good agreement with experimental data.

V. FUTURE SCOPE

- Recombinant strains or mutated strains may be used to increase the keratinase production with less production cost; mixed co-culture can be tried for mixed substrates to improve the production of keratinase enzyme.
- Continuous production of keratinase using immobilized packed bed or fluidized bed reactor may be attempted in order to increase the productivity.

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- Additional researches have to be done for purification and characterization of keratins, studying the kinetics of enzyme testing for the range of substrates, effect of inhibitors and enhancing the activity of keratinase, solid state fermentation and large scale production of keratinase.
- Further work may be extended towards the identification of keratinase - coding gene and the generation of genetically engineered microorganism carrying the identified keratinase gene to increase enzyme production.

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