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Optimization of Cultural Conditions for Keratinase Production by *Curvularia lunata* (JK17) using Response Surface Methodology

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Abstract

This study presents optimum cultural conditions for the maximum keratinase production in SSF applying response surface methodology. The maximum enzyme activity of 123 U/mL for Curvularia lunata was observed at cultural condition 12.72 days incubation, temp. 35^{o}C, 5.6 g feather, 4.9 g carbon, 5.9 g nitrogen. Three-dimensional response surface and contour plots were drawn to find the relationship between keratinase production and cultural conditions. These values optimum for keratinase production by *Curvularia lunata* were in good accord with the experimental result.

Keywords: Keratinase; *Curvularia lunata;* RSM; Submerged state fermentation.

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1. Introduction

The global chicken meat processing business is growing at a tremendous speed. The quick growth rate of chickens and an efficient feed-to-weight ratio are two factors that contribute to the rapid speed. Poultry products are a good source of nutrition for people yet have low economic worth [1]. Feathers are a waste product produced in huge quantities by these poultry industries, constituting 5-7 % of the chicken's weight. Annually millions of tons of chicken feathers are produced worldwide [2,3]. Feathers are made up of keratin protein and dry matter. This byproduct represents a potential substitute to more expensive dietary ingredients and is hard to degrade due to the disulfide bond [4]. Keratinases is an enzyme hydrolyzing keratin, which is an insoluble protein [5]. Keratinases are serine or metalloproteases capable of keratin degradation [6]. However, due to their recalcitrant nature application of feather products is limited. Keratin protein was extracted from chicken feathers and characterized for their quality [7]. Treatment of keratin waste has adverse effects on the atmosphere. That is why the industrial application of keratinase is necessary for tanneries [8], while physical treatment requires energy and

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depletes amino acids. Biodegradation of keratin protein is a process of managing keratinous solid waste for a sustainable environment and byproducts. Keratinolytic enzymes are gaining importance because of are industrially applicable in many industries as detergents, leather processing, biofertilizer development, and bioremediation [9].

Response surface methodology is a set of mathematical and statistical approaches for modeling and investigating issues in which a variable influences response of interest to optimize that response. This process optimization technique supports Taguchi's theory while also providing an easier and more proficient way to understand and implement this concept in practice [10].

Keratinase production using response surface methodology has been reported earlier by bacteria, actinomycetes, and fungi. Few reports of application of RSM in keratinase production are as follows bacteria *Bacillus subtillis* [11], *Bacillus cereus* [4,12], *Actinomyces fradiae* [13] *Bacillus* sp. [14], *B. subtilis* [15], *B. aureus* [16] and fungi *Acremonium strictum* [17], *Scopulariopsis brevicaulis* [18], *Aspergillus* sp. [19]. Because of their extracellular secretion, fungal keratinases are simple to get, and their low cost makes them desirable, even if fungus grow slowly [20].

According to the available literature, no research has been done on optimizing media composition or cultural conditions for keratinase production by *Cuvularia lunata*. The goal of this study was to use a five-level, five-factor central composite rotatable design to optimize culture conditions such as incubation duration, temperature, substrate quantity, nitrogen source, and carbon source for the synthesis of keratinase using RSM. A second-order model was used to construct the three-dimensional response surfaces for synthesis.

2. Materials and Methods

2.1. *Microorganism*

C. lunata JK17 was procured from the departmental culture collection center and subcultured on potato dextrose agar (Fig. 1). Fungal Isolate was previously isolated by the hair baiting method and identified as *Curvularia lunata* by morphological characters and deposited in the departmental collection as isolate JK17. Seven days old cultures of the fungi were used as inoculum.

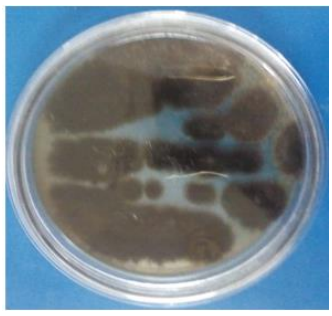

Fig. 1. Growth of *Curvularia lunata* on potato dextrose agar.

2.2. *Culture media*

Keratinase production media were prepared by the method [10, 21], which contained 5 g whole chicken feather, 5 g yeast extract, 1 g K_2HPO_4, 3 g KH_2PO_4, 1 g $CaCl_2$, 1 g $MgSO_4$ in 1000 mL. The above medium was dispensed in a 250 mL Erlenmeyer flask and sterilized by autoclaving at 121 °C for 20 min. Each flask was inoculated with 7 days old culture and incubated at 28±2 °C. The culture broth was obtained through filtration and purified by ammonium sulfate precipitation followed by gel filtration chromatography by Kumar and Mahal [22] and used for the assay.

2.3. *Keratinase assay*

Keratinase production was observed by the method [10,23]. Chicken feather (substrate) 20 mg, 4 mL of buffer (Glycine-NaOH pH 10), 1 mL of the keratinase enzyme were mixed and incubated at 60 °C for 60 min. After incubation, 4 mL of 5 %, TCA was added to stop the reaction and were incubated at room temperature for 30 min. Contents of the test tubes were filtered and centrifuged at 5000 rpm for 10 min at 4°C. In control, 3 mL of Glycine-NaOH buffer (pH 10) was added. The absorbance of the supernatant was observed at 280 nm by UV-VIS spectrophotometer.

2.4. Experimental Design

It is required to choose an experimental design, fit an appropriate function, and assess the standard of the fitted model and its accuracy to make a prediction based on experimental data. Response surface methodology (RSM) is a statistical approach for modeling and analyzing situations in which numerous variables impact response of interest, and the goal is to maximize that response. The current study is predicated on the idea that keratinase production is functionally linked to the technique variable. It attempts to fit a multivariate analytic equation characterizing the response, i.e., *P*. The variables in Table 1 are listed in descending order of presumed significance as process variables.

Central composite design (CCD) was used to investigate the quantitative link between response performance and technique in keratinase synthesis. A complete factorial or fractional factorial design; an additional design, generally a star form in which experimental points are at a distance from the center; and a central point are the components of Box and Wilson's design. All parameters have been investigated at various levels, namely -2,-1,0,+1,+2. A 2^2 full factorial central composite design experiment was performed [25]. The variables were coded according to Eq. 2 for statistical analysis [14,26-28].

$$X_i = \frac{x_i - x_{io}}{\Delta x_i} \quad \text{----------} \tag{2}$$

Where X_i is the coded value of the independent variable, x_i is the real value of an associated independent variable, x_{io} is the actual value of the independent variable on the center point, and Δx_i is the step change value.

The design is determined by a symmetrical selection of variation increments for the core composition. Because the interpretation of the results was legitimate within the experimental limitation, this degree of variation was chosen to be within a tolerable range. In addition, the quantity indicated backed up the findings of earlier research. Table 1 shows the increments of variation for each variable spread around the center point and the mathematical connection between the actual and coded ratios.

Table 1. Variables and their levels for central composite design.

Independent Variables	Symbols		Levels				
	Coded	Actual	-2	-1	0	+1	+2
Incubation Period (days)	X_1	x_1	4	8	12	16	20
Temp (°C)	X_2	x_2	20	25	30	35	40
Amount of substrate (g)	X_3	x_3	3	4	5	6	7
Carbon source (g)	X_4	x_4	1	2	3	4	5
Nitrogen source (g)	X_5	x_5	4	4.5	5	5.5	6

As shown in Table 2, a collection of 30 experiments was carried out. All variables were taken at a central coded value set at zero. The min. and max range of all variables and full experimental plan with respect to their values in the coded form are also listed in Table 2. Keratinase production was taken as a response (Y). A second-order polynomial equation was then fitted to data by a multiple regression procedure.

2.5. *Data analysis*

Multiple regression analysis was conducted for fitting the model described by equation to the experimental results. Maximization or diminution of polynomial so fitted was performed by a numerical technique, using the mathematical optimizer procedure of Quattro Pro 12 of Word office 12 (/s Corel corporations, USA) that deals with constraints. The mapping of the fitted response was achieved using STATGRAPHICS Centurion XV version 15.1.02 (M/s standpoint Inc., USA). The response surfaces contour plot for these models was plotted as a function of 2 variables, whereas keeping extra variables at optimum level.

Table 2. Central composite design arrangements and response.

Experiment no.	Variable Levels					Keratinase production (p)
	X_1	X_2	X_3	X_4	X_5	
1	0	0	+2	+2	-2	45.7
2	+2	0	+2	+2	+2	106.9
3	-1	0	-2	-1	0	84.0
4	-1	+1	-2	+1	0	84.6
5	+2	0	-1	+2	+2	72.6
6	+1	0	0	0	+2	58.9
7	+1	+1	-2	+2	+2	91.1
8	+2	+1	0	+2	+2	151.4
9	0	0	+1	-2	-1	101.6
10	-2	-2	0	0	0	52.3
11	-2	-2	-1	0	0	62.8
12	+2	-2	-2	+2	+2	76.6
13	-2	-2	+2	+2	-2	96.7
14	-1	-2	+2	+2	-1	61.9
15	-1	-2	+1	+1	-2	78.8
16	+2	+2	-1	0	-1	148.2
17	-2	+1	+1	-1	-2	135.8
18	-2	0	-2	+2	-2	47.4
19	-2	+1	-1	+2	-1	75.3
20	-1	+2	+2	-1	0	141.0
21	+1	+2	0	-2	+2	49.1
22	0	+2	-2	+2	+1	71.8
23	-2	+2	+1	0	-2	77.8
24	-2	+2	+2	+1	0	53.1
25	-2	-1	+1	+2	-2	41.4
26	-1	-1	+2	+1	0	80.4
27	0	-1	-2	0	+1	68.4
28	+1	-1	-1	-2	0	73.0
29	+1	-1	-2	+1	+2	68.7
30	+2	-1	0	-1	-1	37.7

3. Results

3.1. *Diagnostic checking of the fitted model*

The coefficient of determination (R_2) is the proportion of variability in the data explained by the diagnostic checking of the fitted model, and larger values of R_2 revealed that this regression is statistically significant. Regression analyses for different models indicated that the fitted quadratic models accounted for more than 96.0 % of the variations in the experimental data, which were found to be highly significant. The experimental data were fitted to a second-order polynomial regression model containing linear, quadratic, and interaction using the same experimental design Software. The regression equation obtained after analysis of variance gives the extent of keratinase production as a function of the different process variables. The significance of all terms is included in the following Eqn.

$p = 0.714994 + (-0.00763X_1 + 0.106317X_2 + 0.0553056X_4 + 0.0194511X_3^2 + 0.076084X_1X_2 + (-0.0110433X_1X_3) + 0.01829063X_1X_5 + (-0.0021X_2X_3) + (-0.042503X_2X_5) + 0.06289117X_4X_52$---------- (1)

Eq. 1 represents an empirical relationship between the yield (*p*) and the independent parameters (X_1-X_5) obtained from response surface methodology modeling. Where *X1*, X_2, X_3, X_4, and X_5 represent coded values of the incubation period, growth temperature, amount of substrate, nitrogen source, and carbon source, respectively, and *p* is the response variable (maximum production of keratinase in unit per 100 mL).

The regression coefficient is presented in Table 3 to determine the statistical significance of the regression model; the F distribution analysis was performed. The analysis of variance for experimental results is shown in Table 4. The analysis of variance of regression model implies the model terms are highly significant.

Table 3. Estimated coefficient of the fitted quadratic equation for keratinase production based on t-statistics.

Coefficient	Estimated coefficient
bk_0	0.71499
bk1	-0.007628
bk2	0.106317
bk3	0.053056
bk33	0.019451
Bk12	0.076084
bk13	-0.110433
bk15	0.018290
bk23	-0.002100
bk25	-0.042503
bk45	0.0628911

3.2. *Analysis of a variance*

When a model is chosen, an analysis of variance is performed to see how well it reflects the data. The suggested model's analysis of variance is shown in Table 4, and the model was evaluated using an *F*-value test. Keratinase synthesis has an *F*-value of 1.821493. On this basis, it is possible to infer that the chosen model properly captures the keratinase production data. According to the study, the residuals were found to be uniformly distributed about zero, with no indication of outliers.

Table 4. Analysis of variance for the proposed model.

Keratinase production	Source of Variation	Df	Sum of squares	Mean square	F	Significance F
P	Regression	20	2.222737	0.111137	1.821493	0.178402
	Residual	9	0.549127	0.061014		
	Total	29	2.771865			

The model proved useful in suggesting which factors to modify in order to optimize keratinase production and the best circumstances to achieve maximal keratinase production (Table 5).

Table 5. Optimum conditions for maximum keratinase production.

Cultural conditions	Coded value	Uncoded value
Incubation time (Days)	0.18	12.72
Temperature (°C)	1	35
Amount of substrate	0.25	5.25
Carbon source	1.9	4.9
Nitrogen source	1.8	5.9

The optimum p-value was higher than the highest value amongst the calculated value based on the experimental design. The surface and contour based on Eq. (2) were prepared with statistical software. The surface plot (Figs. 2-10) shows the behavioral change with respect to simultaneous change into variables. The response surface in Figs. 2-10 is based on the second-order polynomial regression model for *p* (Eq. 2) with three variables kept constant at optimum level and variating the remaining two within the experimental range. The behavior of keratinase production with respect to change in incubation time and temperature at specific values is shown in Fig. 2. An increase in incubation time and decreased temp beyond the optimum value of extent of conversion increased the value of *p*.

Further, at a fixed level of incubation time, the change of p showed a polynomial pattern with incubation time and amount of substrate vice versa (Fig. 3). The change of p showed the linear pattern with incubation time and carbon source (Fig. 4). A similar effect was observed with the carbon source of media and temperature (Fig 5). The amount of substrate changed the extent of conversion in parabolic pattern with temperature (Fig. 6). Also, at a fixed level of temperature, the effect of nitrogen source on *p* was found to be uniformly increasing. Fig. 7 showed the surface and contour plots of *p* as a function temp and nitrogen source, whereas Fig. 8 demonstrated the effect of the substrate with carbon source on the extent of keratinase production (*p*). Fig. 9 has a parabolic pattern that indicates the surface plot between the amount of substrate and nitrogen source regarding keratinase production (*p*). Change of nitrogen source with a carbon source (Fig. 10) demonstrated that the extent of conversion increased progressively with a nitrogen source.

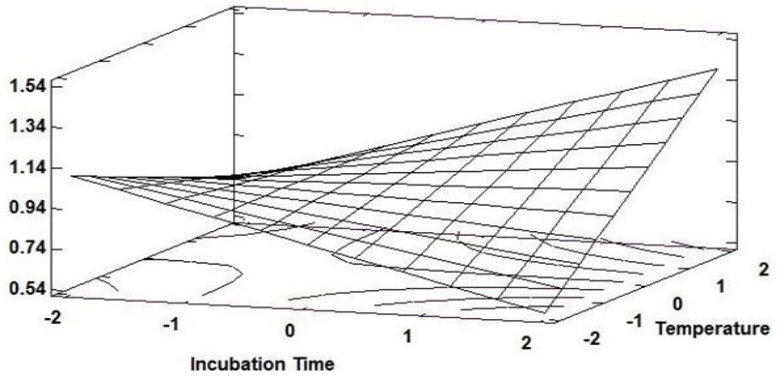

Fig. 2. Surface and contour plot between incubation time and temperature.

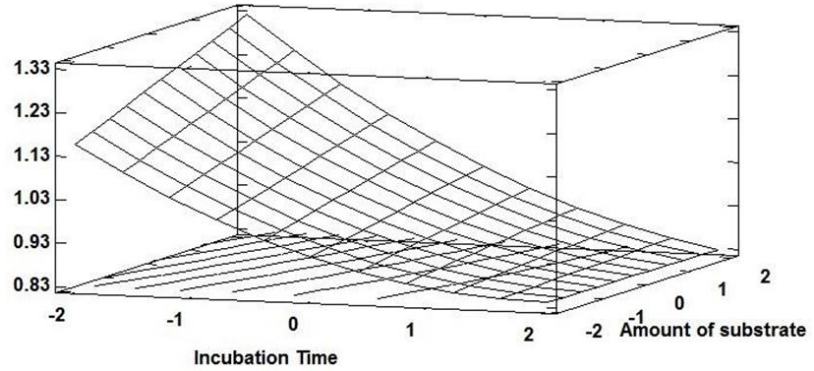

Fig. 3. Surface and contour plot between incubation time and amount of substrate.

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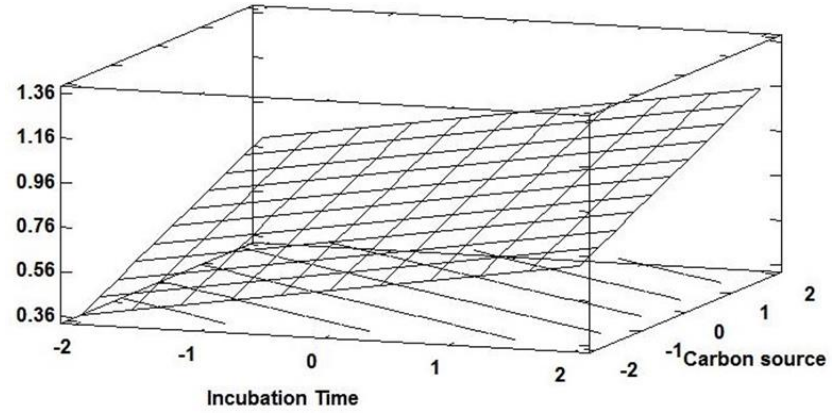

Fig. 4. Surface and contour plot between incubation time and carbon source.

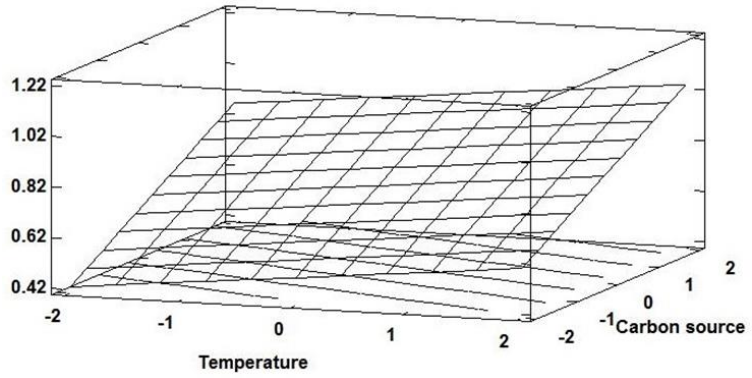

Fig. 5. Surface and contour plot between temperature and carbon source.

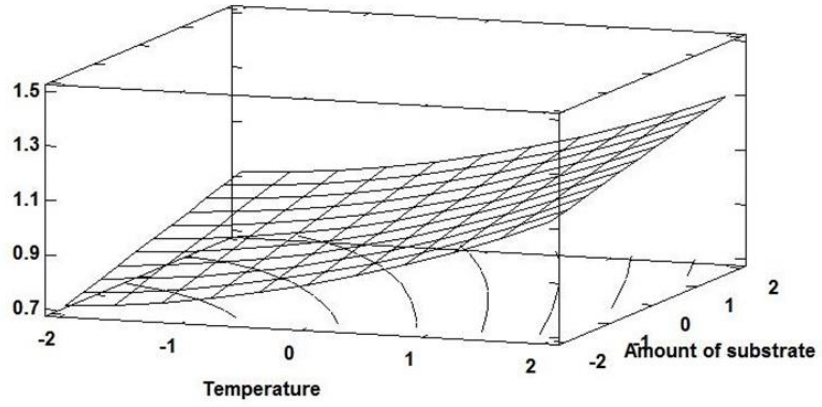

Fig. 6. Surface and contour plot between temperature and amount of substrate.

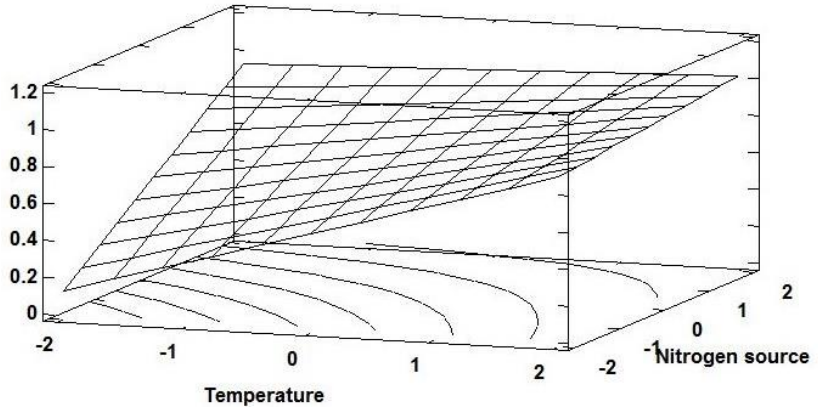

Fig. 7. Surface and contour plot between temperature and nitrogen source.

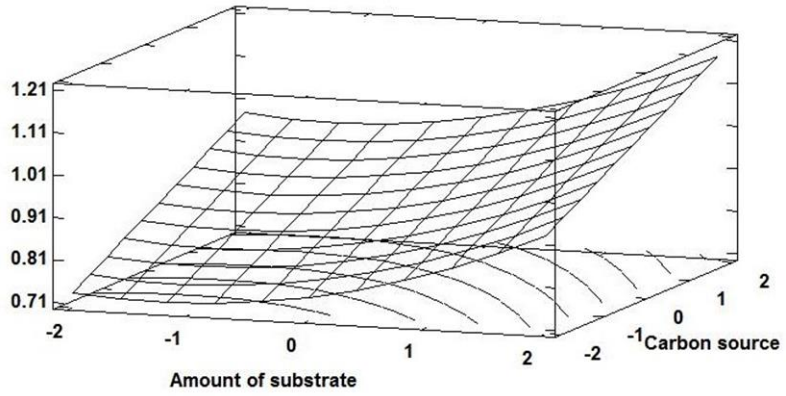

Fig. 8. Surface and contour plot between the amount of substrate and carbon source.

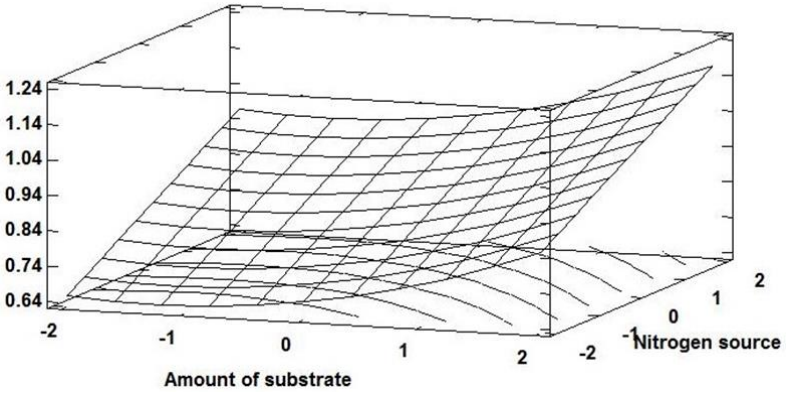

Fig. 9. Surface and contour plot between the amount of substrate and nitrogen source.

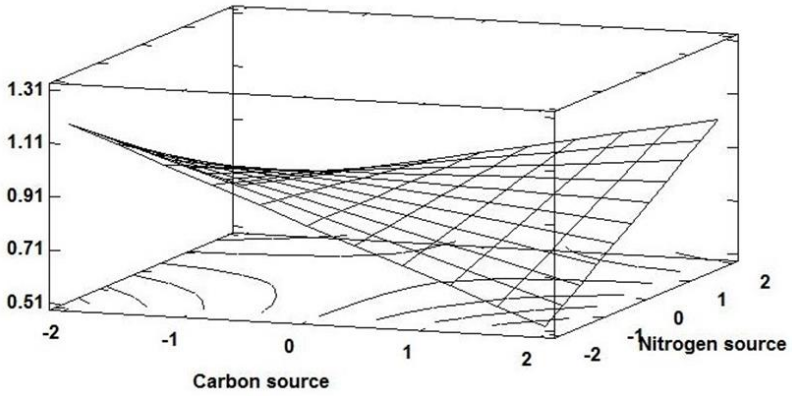

Fig. 10. Surface and contour plot between carbon source and nitrogen source.

4. Discussion

Ramnani and Gupta [10] used the Plackett-Burman design and response surface techniques approach to obtain a 3.5 fold increase in keratinase output by bacterial isolates *Bacillus licheniformis*. [29] Screened out bacteria *Bacillus licheniformis* and was found maximum enzyme activity was 107.6 U/mL. Arokiyaraj *et al*. [12] optimized fermentation condition using *Bacillus cereus* and obtained 292 U/g while *Bacillus aerius* NSMk2 achieved 318 U/mL of keratinase [14], *B. subtilis* was used for keratinase production, and 1.7 fold increase was achieved [15]. Matikeviciene *et al*. [13] optimized parameters and enhanced 46 % keratinase production by *A. fradiae*. The media composition for *Scopulariopsis brevicaulis* keratinase production improved using glucose and soya bean meal as carbon and nitrogen sources 24.8 U/mL and 36.4 U/mL of keratinase activity without using glucose and soya bean meal RSM. Productivity was improved to 225.0 U/mL after using RSM to optimize glucose, soya bean meal, feather powder, and inoculum concentrations, which was a 6.18 time increase over conventional techniques [15]. Bacterial culture *Bacillus* sp. produced 56.218 U/mL keratinase with conditions, i.e. 25 °C temp., 5.84 pH, 5.0 (v/v) bacterial innoculum, 4.97 (w/v) substrate concentration (feather) [16]. In the present study 12.72 days incubation, temp. 35 °C, 5.6 g feather, 4.9 g carbon(glucose), 5.9 g nitrogen (peptone) conditions were optimized, and 123 U /mL keratinase activity was observed.

5. Conclusion

The maximum keratinase enzyme activity 123 U/mL was observed for *C. lunata* JK17 when fermentation is done with the following cultural condition 12.72 days incubation, temp. 35 °C, 5.6 g feather, 4.9 g carbon, 5.9 g nitrogen. This could contribute to the degradation mechanism to control keratinous waste. This paper suggests that optimizing feather degradation and keratinase production could solve the problem of keratinous solid waste landfilling conversion into a high-value product like keratinase, amino acids, animal feed, and slow nitrogen fertilizers.

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