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Optimization of Keratinase Production Using *Pseudomonas aeruginosa* SU-1 Having Feather as Substrate

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Abstract: In this optimization study, *Pseudomonas aeruginosa* SU-1 was producing keratinase at optimal condition of 4 days, pH – 7 and temperature 37 ^{0}C, where it was producing 23.7 U/mL After the one factor at a time, RSM was performed to understand the combination of the physical parameter that ends up for the maximum production of keratinase enzyme and the degradation percentage. The study involved in three variables (pH(A), temperature(B) and Incubation Design (C)) in three ranges (-1,0,+1) using Box-Behnken Design (BBD). The results of the analysis of variance and regression analysis of the second order model showed that the factorial effect if the degradation. The optima of the variables pH - 7, temperature - 30 and incubation time – 4 days. The isolated *Pseudomonas* species was subjected to feather degradation for 4 days and it was degrading 55.26 %. Keratinase was to be size of 56KDa.

Keywords: *Pseudomonas aeruginosa* SU-1; Keratinase; Biomass; Feather Degradation.

1. Introduction

Chicken is one of the major sources of protein for non-vegetarian lovers. During the processing of chicken bulk quantities of feather gets discarded on the barren lands, roadsides, dump yards etc. In many of the developing countries like India it causes public disturbances like dust pollution, bad odour, files and rodents [1]. As the freshly disposed feather carries blood and few pieces of meat which facilitates the growth of various pathogenic microorganisms which emits various toxic gases such as nitrous oxide, ammonia and hydrogen sulphide, which is a major threat for life [2], Feather waste has also suggested being a cause of H5N1 virus outbreaks [3]. Over a period of time these wastes are burnt to reduce the solid waste by the local people which also leads to air pollution, water pollution, the introduction of contaminants in water bodies, creates health problems such as asthma, skin infections etc [4-8] These waste needs proper management to reduce such pollutions.

Since feather is made up of more than 90% of a protein called keratin which is composed of various aminoacids such as cystine, glutamine, lysine, proline, serine, threonine,

and valine [9] and minerals like nitorgen (N), phosphorus, potassium (K), calcium(Ca), magnesium (Mg), iron (Fe), manganese (Mn), zinc (Zn), and copper (Cu) [2] which is potentially good to be used in several fields [10]. Hydrolysed feather products can be used for the production of feed or fodder [11] good nitrogen fertilizer [12] for microbial growth [13].

Feathers can serve as important carbon and nitrogen sources for microbial culture [14]. Traditionally Feathers are treated by steam and chemicals to convert it into feather meal [15], but this method requires more energy and at the same time some of the amino acids may get destroyed during treatment [16]. Many microorganisms are capable of producing a variety of enzymes [17-19] including keratinolytic and proteolytic enzymes which are able to degrade feather keratin [20]. Researchers have isolated various keratinase producing microorganisms from different environments that are rich in keratin and which has been applied for degrading feather waste [21]. In the present study *Pseudomonas aeruginosa* SU-1 was used and optimal conditions such as time, temperature for enzyme production in the powdered feather as carbon source was determined and its effectiveness in full feather degradation was also analyzed.

2. Materials and Methods

2.1. Isolation and Preparation of Inoculum.

Soil sample isolates *Pseudomonas aeruginosa* SU-1 (Genbank accession number - GU395985) was used for this study. All the chemicals used in this study were analytical grade and obtained from Qualigens Chemicals, India.

2.2. Processing of feather.

Broiler chicken feathers were collected from a nearby slaughterhouse in and around Alathur, Kerala, India. Bloodstains and other stains were washed off with tap water. The washed feathers were dried and then boiled in water at 100 ^{0}C for 30 min. after that, it was dried inside hot air oven at 60 ^{0}C. The dried feathers were blended into powder and sieved. The collected powder was used as source of keratin in this study.

2.3. Optimization of growth on feather containing medium.

2.3.1. Incubation period optimization.

Production media was prepared by dissolving K_2HPO_4 (0.04g), feather (0.1g), $MgSO_4$ (0.2g), K_2PO_4 (0.5g), pH – 7.0 in 100 mL distilled water and sterilized under autoclave at 121^{0}C for 15 min. 24 h old culture was inoculated into the media incubated at 37°C for 24 h - 96 h. The enzyme activity was performed using keratinase assay at a different time interval to determine the maximum enzyme activity.

2.3.2. pH optimization.

At 30 ^{0}C temperature, the optimum pH was determined by incubating the chicken feature at different pH ranging from 4,5,6,7,8,9 and 10. The enzyme activity was performed using keratinase assay.

2.3.3. Temperature optimization.

At optimized pH the optimum temperature was determined by incubating the chicken feature at a different temperature ranging from 20, 25, 30, 35, 40 and 45 ^{0}C. the enzyme activity was performed using keratinase assay.

2.3.4. Response Surface Methodology Studies.

A RSM-BBD was conducted to analyzed the specific composition of physical variables required to enhance the production of keratinase enzyme from the chicken features by *Pseudomonas aeruginosa* SU-1. The statistical software package Design expert Version 7.0.0 was used for regression analysis to create different 3D graphs such as surface plot along with contour plot used to assess the statistical variables.

In the beginning of the statistical studies, the best minimal media combination was optimized for the production that conceived as production media. This media provides the basic requirement for the organism to grow and make mandatory of the supplemented compound to be used. The levels of three physical variables (pH, Temperature and incubation time) were selected and each variable are coded A, B and C respectively at three level (-1,0 and 1) Table 1. In the analysis two responses were tested the degradation percentage (%) and the keratinase activity (IU/ml) (Table 2).

The mathematical connection of the independent parameters and the response was calculated by the second-order polynomial equation

$$Y=\eta_0 + A\ \eta_1 + B\ \eta_2 + C\ \eta_1 + AB\ \eta_{12} + BC\ \eta_{23} + AC\ \eta_{13} + A^2 + B^2 + C^2$$

in which Y is the response (Y1 – Degradation percentage and Y2 – Keratinase production) η_0 is the intercept, η_1, η_2, and η_3 are linear coefficient η_{12}, η_{23} and η_{13}.are interaction coefficients.

2.4. Enzyme assay.

5mg keratin azure powder was dissolved in 1mL 50mM Tris HCl buffer (pH 8.0). Now the suspension was added with 1mL of crude enzyme (culture supernatant obtained after centrifugation at 10000 rpm) and kept at 50 ^{0}c for 30 min. following that, 2 mL 0.4 M TCA was added to stop the reaction and centrifuged at 5000 rpm for 40 min, supernatant was read at 590nm. One unit (U) of enzyme activity was defined as described by Cai et al [22].

2.5. Percentage of degradation of chicken feather.

Percentage of feather degradation was found for the organism by sterilizing a feathers weighing 10 g and added to the aforementioned 100 mL media added with 10 % 24 h old *Pseudomonas aeruginosa* SU-1. The set up was done with four steps and a control was also kept, where no organism was inoculated. Every two days gap, media from conical flask was removed and the feather was dried to know the degradation. Thus, it was done for 5 days and percentage degradation was determined.

2.6. Characterization of enzyme.

PAGE (polyacrylamide gel electrophoresis was carried out to identify the molecular weight of the enzyme). Glass plate was assembled space aid agar was used to seal the plate and the percentage separating gel was prepared with 30% of amide, 1.5 M tris buffer, 10% SDS, 10% ammonium per sulfate (APS), TEMED and allowed to polymerization at room

temperature for 2-5 minutes and prepared stacking gel with same chemicals except for 1.5M tris buffer and used 1M tris buffer and the comp was placed immediately and allowed to polymerization. After the comp was removed and the plate with gel was set to electrophoresis units and the SDS tank buffer was added immediately. Prepared sample (10µl of sample and 10µl of 2x SDS stain and incubated at 90°c for 10 minutes) was loaded (10µl) to the well along with marker. Run the gel with 50volt (v) of current for 4 hours, until the bromophenol blue reaches the bottom of the gel. The gel was suspended from the plate and stained with 0.5% commassie brilliant blue in water, acetic acid, methanol in the ratio 25:10:45 in the shaker 60-70 rpm for overnight followed by destained (water :acetic acid : methanol-25:10:40) and the result was observed by using gel documentation and the molecular weight was identified.

3. Results and Discussion

Soil isolate *Pseudomonas aeruginosa* SU-1 used in this study was found to produce more enzyme quantity at 4th day of incubation and the enzyme activity was 45 U/mL (Fig.1), where the optimal pH and temperature was found to be 7 and 30 ^{0}C respectively (Fig.2 and Fig.3). Very few works were reported on the ability of feather-degrading Gram negative bacteria [23-27]. Mohamad et al [24] reported that optimal production of keratinase by *Pseudomonas* sp LM19 at pH 8 and 30 ^{0}C while using 1% feather as substrate. *Pseudomonas* sp., MS21 was producing maximum at 37°C and pH 8.0 [23].

The results obtained in terms of degradation percentage by suggesting to the experiments executed conferring to the BBD are given in Table 3. In the regression analysis, coefficient of determination (R^2) was computed as 0.980099 for Degradation percentage production (model summary in Table 4), indicating that the statistical model can explain 98.0% of variability in the response. The adjusted R^2 value corrects the R^2 value for the sample size and for the number of terms in the model. The value of the adjusted determination coefficient (Adj R^2 =0.954512) is also very high, sponsoring higher importance of the model. R2 value calculated as 0.983097 for keratinase production indicating the statistical model can be 98.3%. The adjusted R2 0.961365 correlates with the R2 value. The predicted R2 value 0.970545 correlates along with the other values.

The empirical relationship between degradation percentage (Y1) and keratinase production (Y2) and the three test factors in coded units obtained by using the formulae

Keratinase activity = 21.8 + 1.362 A - 0.925 B+ 0.3625C - 3.55AB + 1.325 AC - 4.55 BC - 8.3625 A^2 -7.0875 B^2 -5.6125 C^2

Degradation % = 50.187+ 3.347 A - 1.849 B + 0.577 C -7.733 AB +2.938 AC-10.38 BC - 18.675 A^2-15.883 B^2- 12.812 C^2

The regression analysis results in the developed x of surface plots that reveal the relationship of the variables with respect to the response. The response (%) of degradation and (IU/mL) of keratinase from chicken feather for different test parameters can also be predicted from the respective surface plots, as shown in Fig. 4 and Fig.5. Each surface plot represents an infinite number of permutation and combination of two test parameters at their corresponding zero level. These plots demonstrate that the degradation and production of keratinase were dependent on the linear effects of pH, temperature and incubation time. The results of physical condition optimization studies from *Pseudomonas aeruginosa* SU-1 used in the present studies were good and compared well with those of studies of other microorganisms. Thus, the condition was optimized with RSM shows at pH 7, Temperature 30 ^{0}C and incubation of 4 days showed maximum productivity and degradation of the chicken feather powder.

In this study, it was found that *P.aeruginosa* SU-1 was degrading 55.26 % of the feather (Fig. 6 and 7). Approximately 86% feather degradation was achieved at optimal condition by *Pseudomonas* sp LM19 [24]. In our study, the organism was degrading up to 55 % in the optimal medium condition in 4 - 5 days. *B. licheniformis* BBE11-1 and *Stenotrophomonas maltophilia* BBE11-1 were able to degrade 35.4% and 22.8% respectively in 96 h. while they were allowed to degrade in a coculture system, it was reaching to 55.2% which might the higher keratinase/protease activities [28]. The organism used in this study was producing enzyme of size 56 KDa (Fig.8), where Han et al [29] reported *P.aeruginosa* C11 to produce 33 KDa sized keratinase and Tork et al [23] found *Pseudomonas* sp., MS21 to produce 30 KDa keratinase. The enzyme produced here might be a dimeric enzyme. These keratin derived hydrolysates have been reported to have more application including phyto stimulant and biofertilizer [30 – 33]. Microbial derived products and their uses are well defined already [34,35].

Table 1. Experimental Range and Levels of three Independent Variables Used in response surface methodology in Terms of low and high, actual and coded factors for Optimization of physical parameters for the Degradation and Keratinase Production as response.

Factor	Name	Units	Low		High		Std. Dev.
			Coded	Actual	Coded	Actual	
A	pH		-1	5	1	9	1.371989
B	Temperature	C	-1	25	1	35	3.429972
C	Incubation time	days	-1	2	1	6	1.371989
Response	Name	Units	Minimum		Maximum		Mean
Y1	Degradation %	%	12.5		94.5		47.7
Y2	Keratinase activity	IU/ml	2.5		23.7		11.88824

Table 2. Design Matrix of BBD and Observed Results for Degration precentage and Keratinase Production.

Run	Factor 1	Factor 2	Factor 3	Response			
	A:pH	B:Temperature	C:Incubation time	Degradation %		Keratinase activity	
		C	Days	%		IU/ml	
1	7	35	2	52.4	30.64	12.4	12.4
2	9	25	4	50.4	29.47	12.3	12.2
3	7	30	4	82.6	48.30	22.3	21.8
4	7	30	4	85.6	50.06	21.4	21.8
5	7	30	4	94.5	55.26	23.7	21.8
6	7	30	4	91.6	53.57	22.9	21.8
7	5	30	6	22.7	13.27	5.4	5.5
8	7	35	6	17.9	10.47	4.2	4.0
9	5	35	4	29.5	17.25	7.5	7.6
10	7	25	6	56.6	33.10	14.9	14.9
11	7	25	2	20.1	11.75	4.9	5.1
12	9	30	6	44.2	25.85	10.8	10.9
13	5	30	2	29.8	17.43	7.5	7.4
14	7	30	4	74.8	43.74	18.7	21.8
15	9	30	2	31.2	18.25	7.6	7.5
16	5	25	4	12.5	7.31	2.5	2.4
17	9	35	4	14.5	8.48	3.1	3.2

Table 3. Model Coefficients Estimated by Multiple Linear Regression for the Keratinase production.

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F	
Model	874.0301	9	97.11446	45.23715	< 0.0001	Significant
A-pH	14.85125	1	14.85125	6.917901	0.0339	
B-Temperature	6.845	1	6.845	3.188488	0.1173	
C-Incubation time	1.05125	1	1.05125	0.489686	0.5066	
AB	50.41	1	50.41	23.48162	0.0019	
AC	7.0225	1	7.0225	3.27117	0.1134	
BC	82.81	1	82.81	38.57395	0.0004	
A^2	294.448	1	294.448	137.1576	< 0.0001	
B^2	211.5059	1	211.5059	98.52214	< 0.0001	
C^2	132.6322	1	132.6322	61.78178	0.0001	
Residual	15.0275	7	2.146786			

Lack of Fit	0.1875	3	0.0625	0.016846	0.9965	not significant
Pure Error	14.84	4	3.71			
Cor Total	889.0576	16				
Std. Dev.	1.465191		R-Squared		0.983097	
Mean	11.88824		Adj R-Squared		0.961365	
C.V. %	12.32472		Pred R-Squared		0.970545	
PRESS	26.1875		Adeq Precision		17.29699	

Table 4. Model Coefficients Estimated by Multiple Linear Regression for the degradation precentage.

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F	
Model	4413.344	9	490.3716	38.30426	< 0.0001	significant
A-pH	89.67033	1	89.67033	7.004394	0.0331	
B-Temperature	27.36269	1	27.36269	2.137375	0.1871	
C-Incubation time	2.667915	1	2.667915	0.208398	0.6619	
AB	239.254	1	239.254	18.68878	0.0035	
AC	34.5414	1	34.5414	2.698123	0.1445	
BC	430.9873	1	430.9873	33.6656	0.0007	
A^2	1468.514	1	1468.514	114.7096	< 0.0001	
B^2	1062.194	1	1062.194	82.97085	< 0.0001	
C^2	691.2401	1	691.2401	53.99465	0.0002	
Residual	89.61407	7	12.80201			
Lack of Fit	7.329606	3	2.443202	0.118769	0.9444	not significant
Pure Error	82.28446	4	20.57112			
Cor Total	4502.958	16				
Std. Dev.	3.57799		R-Squared		0.980099	
Mean	27.89474		Adj R-Squared		0.954512	
C.V. %	12.82676		Pred R-Squared		0.945404	
PRESS	245.8432		Adeq Precision		15.95767	

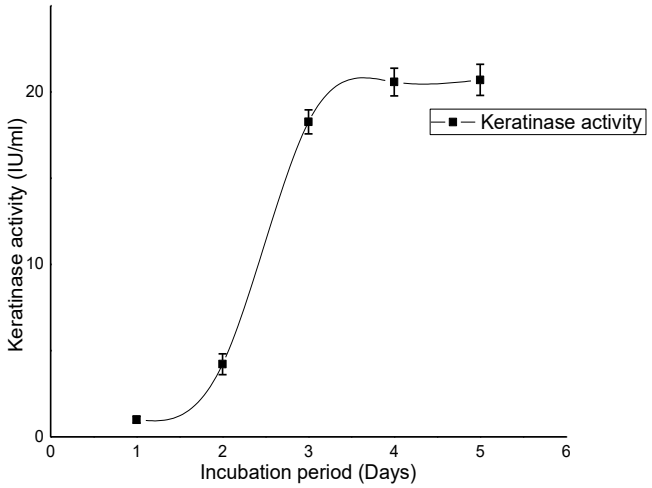

Figure 1. Keratinase activity of *P.aeruginosa* at different incubation time.

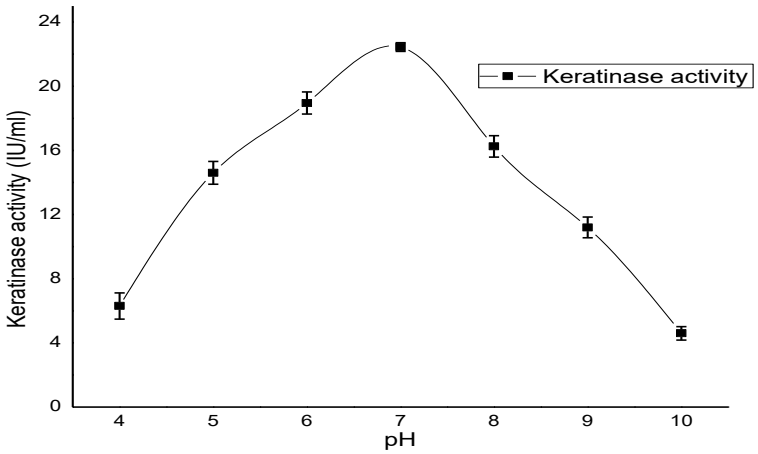

Figure 2. Keratinase activity of *P.aeruginosa* at different pH.

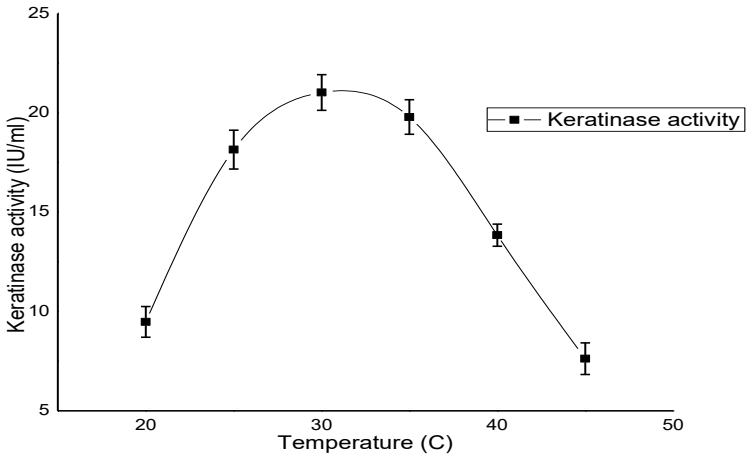

Figure 3. Keratinase activity of *P.aeruginosa* at different temperature.

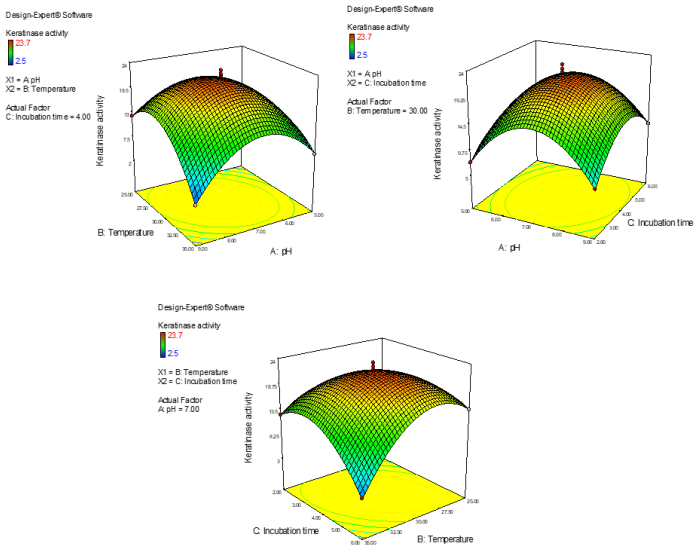

Figure 4. Surface plots of keratinase production optimization studies. **(A)** Effect of pH and temperature and their interaction on keratinase production; **(B)** effect of pH and incubation period and their interaction on keratinase production; **(C)** effect of Temperature and incubation period and their interaction on keratinase production.

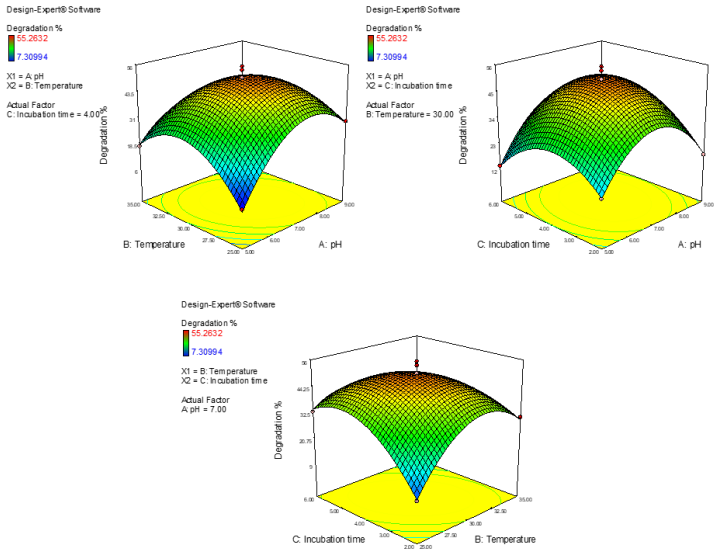

Figure 5. Surface plots of keratinase production optimization studies. **(A)** Effect of pH and temperature and their interaction on keratinase production; **(B)** effect of pH and incubation period and their interaction on keratinase production; **(C)** effect of Temperature and incubation period and their interaction on keratinase production.

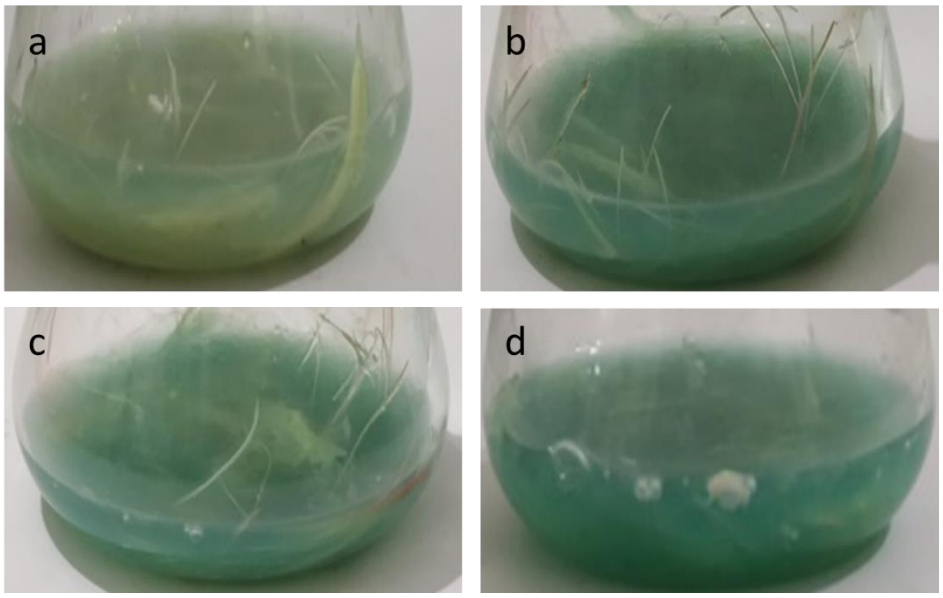

Figure 6. Percentage degradation of feather by *Pseudomonas aeruginosa* SU-1 a) Day – 2, b) Day – 3, c) Day – 4 and d) Day – 5.

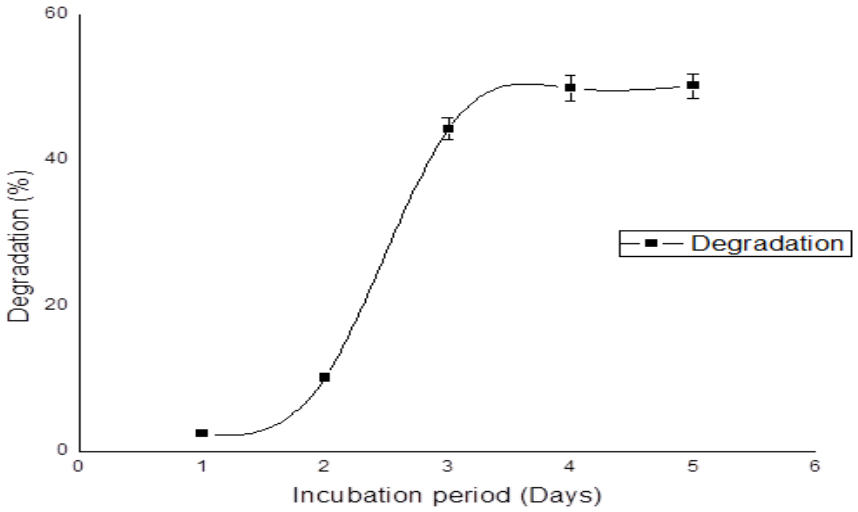

Figure 7. Feather degradation by *Pseudomonas aeruginosa* SU-1 a) Day 2 b) Day 4 c) Day 6 and d) Day 8.

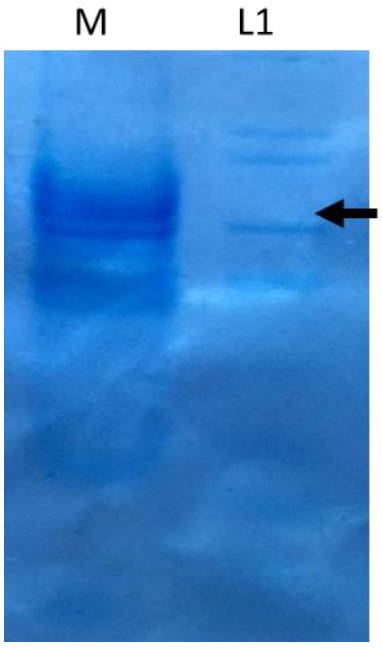

Figure 8. SDS – PAGE analysis of keratinase M – Marker, L1 – Lane 1 Keratinase enzyme (56 KDa).

4. Conclusions

In this study, soil isolates *Pseudomonas aeruginosa* SU-1 was used to produce keratinase having feather powder as source, where the optimal condition was determined as pH-7, temperature - 30 ^{0}C and incubation time – 4 days. The organism was degrading 55 % of the feather in 4 days. Keratinase produced by the organism was about the size of 56KDa.

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This research has no ackwnoledgments.

Conflicts of Interest

The authors declare no conflict of interest.

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