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"Revolutionizing The Enhancement Of Nutritional Conditions In Bioprocessing To Achieve Peak Keratinase Output By *Grass Bacillus* RAS04 In A Sequential Approach."

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Abstract:
A keratinase is an extracellular protease capable of efficiently hydrolysing hair and spine keratin into essential amino acids. It has important applications in animal feed as well as slow nitrogen-releasing fertilizer. Extensive keratinous waste soil screened for alkaline proteases and among them selected potent isolates based on REA. Further analysis of keratinase producers using feather degrading efficiency in the basal salt medium. An efficient keratinase analysis of morphology, culture, and biochemistry coupled with 16S rRNA sequencing was used to select and identify the producer as a *Grass Bacillus* RAS04. An optimization strategy was formulated utilizing "Plackett-Burman design (PBD) and Central Composite Design-Response Surface Methodology (CCD-RSM)" for modulation of nutritional parameters in the bioprocess. The intentionbe to maximize the creation of keratinase from *Grass bacillus* RAS04 (OQ519653.1). PBD was used to screen out eight variables involved in the processing process.Several factors (such as yeast extract, glucose, feather meal, and K2HPO4) In order to further optimize the variables found to be important to keratinase production, the RSM was fitted with a central composite design. According to the results, 72 hours of incubation in a modified basal salt medium with glucose 0.278%, yeast extract 0.695%, feather meal 0.975%, and K_2HPO_4 0.121% resulted in positive results, keratinase enzyme activity was 234.41 units/mL, a 2.38-fold increasein activity over the basal medium.

Keywords: Keratinous waste, keratinase, optimization, Placket- Burkman design, central composite design, etc.

Introduction:

Keratin-containing materials, abundant in nature, pose challenges due to their insolubility, limiting practical applications. Feather waste, estimated at 8.55 million tons annually, is conventionally disposed of through incineration or low-quality animal feed conversion, but these methods have limitations. Heat-sensitive amino acids in the feed are destroyed during incineration, and higher energy consumption is required for feed conversion. In contrast, biotechnological approaches offer alternatives that address nutrition without affecting keratin waste.

Feather waste, a by-product of poultry processing, contains approximately 90% keratin, making it a valuable protein resource. However, conventional proteolytic enzymes like pepsin, trypsin, and papain cannot degrade keratin, leading to its disposal and potential environmental hazards. Notably, keratinases produced by certain microorganisms, such as Bacillus cereus HD1 and various fungi and actinomycetes, can degrade keratins. These enzymes have diverse characteristics, and their biosynthesis holds promise for various applications, including animal feed, fertilizers, detergents, leather, and pharmaceuticals.

Despite their potential, commercial keratinases have limitations in terms of organic solvent tolerance and industrial applicability. Developing new enzymes with improved properties is crucial for commercial viability. Studies have shown that unpolished keratinase from Bacillus licheniformis PWD-1 enhances poultry growth, highlighting its potential as a feed additive. Additionally, keratinase can degrade infectious prion forms like PrPsc in the presence of detergents and temperature, which could have implications for utilizing animal meals as food sources and addressing prion-related concerns.

In the leather industry, keratinases from strains like Grass Bacillus S14 offer eco-friendly options for dehairing leather without damaging collagen. However, optimizing fermentation conditions, including strain selection and media composition, is essential for maximizing microbial enzyme production. Traditional methods involve screening and optimizing one factor at a time, which is time-consuming and lacks consideration of interactions between variables.

To address these challenges, a sequential and strategic approach was adopted in this study. Plackett-Burman designs were initially used to screen and identify significant factors influencing keratinase production. Subsequently, response surface methodology and composite design tools in Design-Expert 13.0.1.0 were employed to further optimize the selected process variables.

Replicate experiments were conducted with Grass Bacillus RAS04 to validate the proposed optimal conditions for maximal keratinase production. This comprehensive approach aims to overcome limitations associated with conventional methods, offering promising avenues for utilizing keratin-containing materials effectively in various industries while addressing environmental concerns.

Materials and Methods:

Materials:

Various suppliers provide a diverse array of reagents, dietary substrates, and chemicals. Merck in Mumbai, India, and SD Fine in Baroda, Gujarat, India, offer these supplies. Additionally, Sigma Aldrich in St. Louis, Missouri, USA, and SRL in East Mumbai, India, are notable sources. For laboratory media, HI Media in Mumbai, India, provides a comprehensive collection. These suppliers cater to the needs of laboratories, offering a wide range of high-quality products essential for research and experimentation.

Microorganism:

After screening over thirteen diverse soil samples containing keratin waste, Grass bacillus RAS04 (OQ519653.1) was discovered. This particular isolate exhibited the highest keratinase production among all tested samples, as indicated by the results of the evaluation.

Conditions for cultivating and obtaining crude enzymes:

The experiment involved utilizing Grass bacillus RAS04 culture in submerged fermentation. Two production media were employed: one modified medium containing 0.5gm of feathers (Rao & Narasu, 2007), and another with 0.5gm feathers in a salt medium (carbon and nitrogen sourced solely from feathers). Fermentation occurred at 37ºC, 120 rpm for 48 hours, using a sterile-modified production medium and basic salt medium, both inoculated with a 3% active bacterial culture. Post-fermentation, the supernatant was collected following centrifugation at 10,000 grams for 10.0 minutes at 4°C, and subsequently analyzed for Keratinase activity.

Keratinase assay:

In the keratinase assay by Cai et al., amino acid (Tyrosine) release was assessed using a modified UV-visible spectrophotometer method (C.-g. Cai et al., 2008). Following the addition of the reaction mixture to tubes, incubation in a water bath at 50°C for 20 minutes was conducted until the reaction was complete. To

terminate the reaction, 10% TCA was added. The addition of the enzyme to the TCA solution after its addition served as a blank. After precipitation at room temperature, centrifugation at 10,000 grams for 10.0 minutes at 4°C was performed to remove the precipitated protein. The supernatant was then evaluated for absorbance at 280 nm using a UV-visible spectrophotometer, indicating Keratinase activity.

$$\boldsymbol{Keratinase\ Activity\ (U/ml)\ =\ 4\ x\ N\ x\ A280/0.01/20}$$

"Were, n - dilution rate, 4 - final reaction volume, 20 - incubation time
In the context of international units (IUs), keratinase is quantified as the enzyme quantity capable of releasing one mole of amino acids per minute per millilitre (mol/mL/min)."

Plackett-Burman design: screening of variable important for Keratinase production:
The determination of this learning is to conclude the conditions that influence the maximum invention of keratinase, Plackett-Burman design [41] was employed for *Grass bacillus* RAS04 According to the literature review, and Process variables were selected based on medium components and environmental factors. In accordance with the design of the experiment, submerged fermentation was conducted. Using Unit/mL as the unit of measurement, the keratinase activity of the samples was determined. A total of twelve consecutive runs of experiments were conducted to select significant variables, seven selected variables and four dummy variables, each of which had two levels (one high level and the otherlow-down level) as exposed in **Table 01**. Within order to maximize keratinase production, we optimized variables with 95% confidence level or higher using the Response Surface Methodology (CCD).

Optimising keratinase production using CCD-RSM and other variables:
Optimising the medium for maximum production of keratinase was accomplished using CCD of RSM. Various levels of analysis were carried out on four variables for the present study, higher (+2),higher (+1), middle (0),lower (-1), and least low (-2) with thirty runs experiment as shown in **Table 02**. A study of the absence of fit and pure error of this model is undertaken for the purpose of studying its pure error; six repetitions of the centre point are made. A set of 2ndorder polynomial equations be provided to elucidate the behaviour of the model.

$$Y = \beta\_0 + \sum\_i \beta\_i X\_i + \sum\_ii \beta\_ii X\_i^2 + \sum\_ij \beta\_ij X\_i X\_j$$

"Inside this context, it is important to clarify that Y denotes the predicted outcome (Keratinase activity). The model incorporates β0 as the steadyexpression, βi as the linear coefficient, βii as the quadratic coefficient, and βij as the interaction coefficient. The independent coded variables are denoted by Xi and Xj, respectively, as per work of Baskar and Renganathan in 2012."
Using Design-Expert software, we conducted a regression analysis. A model with a large degree of appropriate, and a p-value lesser than 0.05 was compared with a model with an insignificant Lack of Fit test. Statistical consequence of the proposed classical calculation was evaluated by means of the appropriate statistics standards of the proposed model terms, including the F value, the coefficient of determination (R^2), and the signal-to-noise ratio, as well as the difference in R^2 values among adjusted and predicted (Montgomery, 1991). Additionally, diagnosis as well as influences of different types of plot results was analyzed to determine whether the suggested model was suitable. Experimental and response variables were analyzed using surface plots and counterplots. In Design-Expert software, the optimization method was used to optimize every variable's level so that maximum keratinase production could be achieved. The model's optimal solution was then validated in experiments. In order to assess the production of keratinase, Plackett-Burman experiments were conducted using independent two-level variables.

Results and Discussion:

"Plackett–Burman design":
Twelve experiments incorporating four dummy variables were conducted using the Plackett-Burman design to assess seven process parameters and identify significant factors affecting keratinase production. Glucose, yeast extract, feather meal, and K2HPO4 were found to positively impact keratinase production, while corn flour, CaCl2, and pH had negative effects. The Pareto chart visually illustrates these effects, with orange

bars representing positive influences and blue bars indicating negative influences on keratinase production. Glucose, yeast extract, feather meal, and K2HPO4 were notably more influential compared to other factors, as evidenced by their larger effects on keratinase production. The ANOVA results confirm the model's significance, with a low probability of obtaining such a high F-value by chance. Glucose, yeast extract, feather meal, and K2HPO4 are identified as significant factors contributing to keratinase production, as supported by their low p-values. These findings underscore the crucial role of these four nutritional parameters in optimizing keratinase production by Grass Bacillus RAS04 in the bioprocess.

"CCD-RSM optimization of selected variables to maximize Keratinase production":

A central composite design (CCD) combined with response surface methodology was employed to optimize the impact of various procedure variables identified in the Plackett-Burman design on keratinase production. Thirty experimental runs were conducted, generating a dataset of responses for each numeric factor at different levels, including six replications at the midpoint. The model's low F-value (81.71) prompted the use of a quadratic model, with noise accounting for only 0.01% of the F-value. The determination coefficient (R^2=0.9871) indicated a strong correlation between measured and predicted responses, with an adjusted R^2 of 0.9750 falling within practical limits. A signal-to-noise ratio of 38.0287 demonstrated adequate precision. Lack of fit analysis revealed a non-significant gap between the model and pure error, supporting the suitability of the quadratic model for enhanced keratinase production. Diagnostic tools, including residual plots and box-cox analysis, confirmed the model's adequacy. The model's equation, based on coded factors and a second-order polynomial, allows for predicting the response of each factor and assessing their relative influence through coefficient comparison.

$$\boldsymbol{Keratinase\ activity} = +192.83 - 12.46\,A + 2.62\,B + 6.13\,C + 63.54\,D + 3.94\,AB - 6.44\,AC - 20.69\,AD - 13.81\,BC + 1.44\,BD - 8.19\,CD - 8.14\,A2 + 2.11\,B2 + 11.49\,C2 + 21.36\,D2$$

In the experimental setup, two numerical factors were varied while keeping other conditions constant at their midpoint values to analyze their combined effect on keratinase production. Optimal keratinase yields were observed at higher levels of yeast extract and midrange glucose concentrations (Figure 03A). Conversely, lower glucose levels and higher feather meal concentrations led to peak keratinase production (Figure 03B). Adjusting K2HPO4 and yeast extract concentrations upward enhanced keratinase production (Figure 03C), while midrange glucose levels with higher K2HPO4 concentrations optimized yields (Figure 03D). Maximum keratinase production occurred at extreme values of yeast extract and feather meal (Figure 03E), and increasing feather meal while decreasing K2HPO4 enhanced production (Figure 03F). Glucose levels within a certain range showed negligible impact on keratinase production.

The optimal conditions for maximizing keratinase production by Grass Bacillus RAS04 were determined using Design-Expert software and validated through laboratory flask experiments. The recommended parameters included 0.278% glucose, 0.121% K2HPO4, 0.975% feather meal, 0.695% yeast extract, pH 9.00, and a development time of 72 hours. Validation experiments confirmed the predicted response fell within the 95% prediction interval range (334.41 Units/ml). The central composite design combined with response surface methodology resulted in approximately a 2.38-fold increase in keratinase production compared to basal medium.

Glucose, a vital component of the medium, exhibited an intricate relationship with keratinase production. Optimal production was achieved at 0.278% glucose concentration, with higher concentrations leading to decreased yields due to catabolite repression. Similarly, yeast extract played a significant role, with concentrations above 0.695% increasing keratinase production but decreasing beyond this point due to catabolite repression.

Feather meal served as the substrate for inducing keratinase production, with 0.975% concentration maximizing yields. However, higher concentrations were found to inhibit keratinase production, potentially due to increased substrate viscosity hindering enzyme expression.

K2HPO4, an essential mineral salt, also influenced keratinase production. Concentrations above 0.121% led to increased production, while higher concentrations resulted in decreased yields. This could be attributed to the differing effects of K2HPO4 on enzyme production across microbial species, necessitating optimization for each species.

Comparison with previous studies highlighted the efficacy of the proposed optimization strategy. For instance, similar methodologies applied to other Bacillus species resulted in notable increases in keratinase yield. The use of mineral salts like K2HPO4 has also been observed to stimulate keratinase production in certain microbial strains.

Overall, the optimization of nutritional parameters, including glucose, yeast extract, feather meal, and K2HPO4 concentrations, is crucial for maximizing keratinase production by Grass Bacillus RAS04, with each component exhibiting complex effects on enzyme expression and substrate utilization.

Conclusion:

Statistical analysis of Grass Bacillus RAS04 in submerged fermentation, utilizing an altered Basal Salt medium, identified glucose, yeast extract, K2HPO4, and feather meal as significant variables influencing keratinase production. Through essential compounds design and response surface methodology, optimal levels of these nutritional parameters were determined, resulting in a maximum keratinase activity of 334.41 Units/mL. Compared to an unoptimized basal salt medium, keratinase productivity increased 2.38-fold. This statistical experimental design minimizes the number of experiments needed to optimize keratinase production. Future applications of keratinase include treating prion diseases, improving animal feed quality, enhancing the leather industry, and bolstering agriculture.

Table01: "Plackett-Burman experimental design for keratinase production using independent two-level variables."

Factors	Name	Units	Low (-1)	High (+1)
A	Glucose	%(w/v)	0	0.05
B	Yeast Extract	%(w/v)	0.1	1
C	Dummy-1		-1	1
D	CaCl2	%(w/v)	0.02	0.2
E	Corn flour	%(w/v)	0	0.1
F	Feather meal	%(w/v)	0.1	0.5
G	pH		7	9
H	K_2HPO_4	%(w/v)	0.05	0.5
J	Dummy-2		-1	1
K	Dummy -3		-1	1
L	Dummy-4		-1	1

Table 02: *"Experimental variables of the CCD-RSM those are coded and uncoded."*

Level	Coded level	Un-coded level			
		Glucose (% w/v)	Yeast Extract (% w/v)	K_2HPO_4 (% w/v)	Feather Meal (% w/v)
Alpha (-α)	-2	0.01	0.05	0.01	0.1
Low	-1	0.0325	0.1625	0.0325	0.325
Mid	0	0.055	0.275	0.055	0.55
High	1	0.0775	0.3875	0.0775	0.775
Alpha (+α)	2	0.1	0.5	0.1	1.0

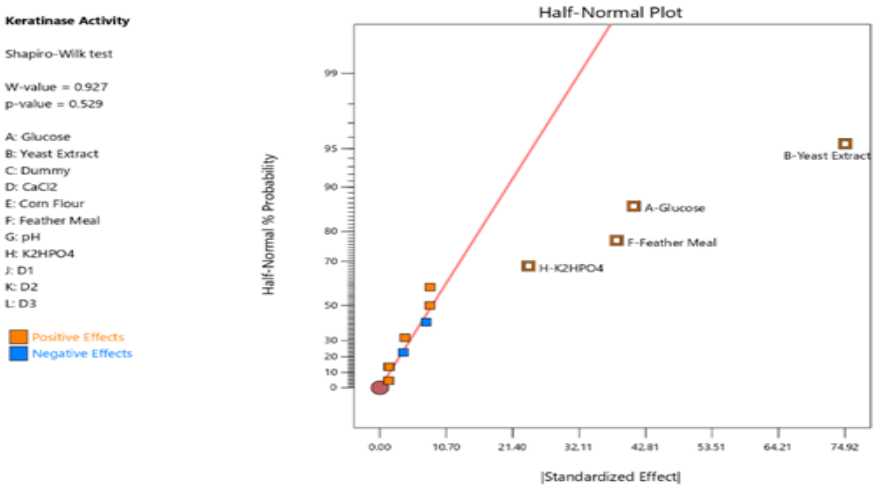


Figure 1. An outlier plot shows a significant effect on keratinase production when compared to a half-normal plot.

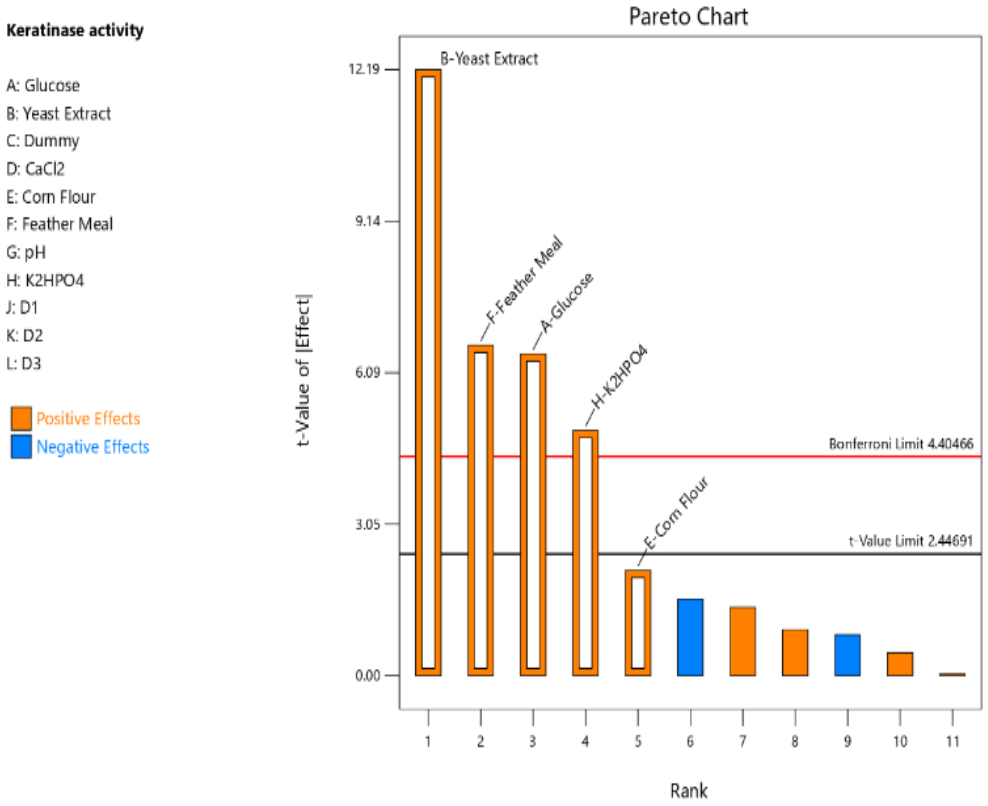


Figure 02: "ThePareto chart of 11 factors showing order of significance."

Table 03: An experimental design for Keratinase Production using a Plackett-Burman method with coded independent variables.

	Factor 1	Factor 2	Factor 3	Factor 4	Factor 5	Factor 6	Factor 7	Factor 8	Factor 9	Factor 10	Factor 11	Response 1
Run	A: Glucose % w/v	B: Yeast Extract % w/v	C: Dummy 1	D: $CaCl_2$ %(w/v)	E: Corn Flour %(w/v)	F: Feather Meal %(w/v)	G: pH	H: K_2HPO_4 %(w/v)	J: Dummy 2	K: Dummy 3	L: Dummy 4	Keratinase activity Units/ml
1	-1	-1	-1	+1	-1	+1	+1	-1	1	1	1	91.3
2	+1	+1	-1	-1	-1	+1	-1	+1	1	-1	1	249.8
3	+1	-1	+1	+1	+1	-1	-1	-1	1	-1	1	115.2
4	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	50.8
5	+1	+1	+1	-1	-1	-1	+1	-1	1	1	-1	164.2
6	-1	+1	+1	-1	+1	+1	+1	-1	-1	-1	1	190.4
7	+1	-1	+1	+1	-1	+1	+1	+1	-1	-1	-1	159.2
8	+1	-1	-1	-1	+1	-1	+1	+1	-1	1	1	132.7
9	-1	+1	+1	+1	-1	-1	-1	+1	-1	1	1	152.3
10	-1	-1	+1	-1	+1	+1	-1	+1	1	1	-1	133.6
11	+1	+1	-1	+1	+1	+1	-1	-1	-1	1	-1	204.1
12	-1	+1	-1	+1	+1	-1	+1	+1	1	-1	-1	169.3

Table 04: "An analysis of the Plackett-Burman Design Experiment statistics":

Source	Sum of Squares	Df	Mean Square	F-value	p-value	
Model	29636.70	7	4233.75	28.36	0.0031	Significant
A-Glucose	4700.56	1	4700.56	31.49	0.0058	
B-Yeast Extract	16673.15	1	16673.16	111.55	0.0009	
D-CaCl2	75.56	1	75.55	0.5048	0.5167	
E-Corn Flour	503.16	1	503.16	3.39	0.1406	
F-Feather Meal	4957.28	1	4957.29	33.19	0.0047	
G-pH	0.1409	1	0.1402	0.0008	0.9778	
H-K_2HPO_4	2727.08	1	2727.04	18.26	0.0128	
Residual	598.16	4	149.56			
Cor Total	30234.84	11				

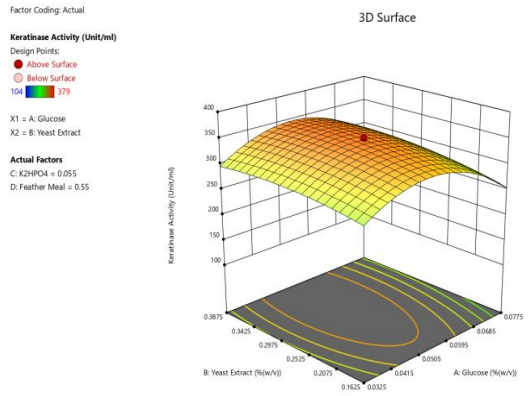
Table 05: The Design layout for the CCD-RSM using four coded variables with experimental and predicted outcomes:

Run	A: Glucose	B: Yeast Extract	C: K_2HPO_4	D: Feather Meal	Experimental Response	Predicted Response
	%(w/v)	%(w/v)	%(w/v)	%(w/v)	Keratinase Activity (Units/ml)	
1	0.056	0.275	0.01	0.55	228	230.524
2	0.0323	0.1625	0.0775	0.325	201	190.223
3	0.0776	0.3875	0.0775	0.775	233	237.06
4	0.057	0.275	0.1	0.55	244	256.093
5	0.0779	0.3875	0.0775	0.325	178	163.094
6	0.052	0.275	0.055	0.55	192	196.12
7	0.056	0.05	0.055	0.55	191	192.257
8	0.0776	0.1625	0.0775	0.325	168	187.318
9	0.0324	0.3875	0.0325	0.325	139	131.093
10	0.12	0.275	0.055	0.55	138	154.024
11	0.056	0.275	0.055	0.55	194	196.12
12	0.0324	0.1625	0.0325	0.325	118	122.516
13	0.056	0.275	0.055	0.55	186	196.16
14	0.0327	0.3875	0.0325	0.775	332	317.88
15	0.056	0.275	0.055	1	409	396.243

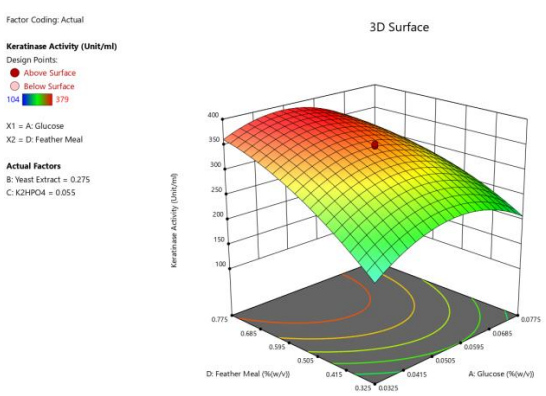
16	0.0777	0.1625	0.0325	0.325	148	153.009
17	0.056	0.275	0.055	0.1	147	150.377
18	0.059	0.275	0.055	0.55	196	196.13
19	0.0778	0.1625	0.0775	0.775	258	246.42
20	0.056	0.275	0.055	0.55	189	196.10
21	0.0778	0.3875	0.0325	0.775	272	279.193
22	0.0776	0.3875	0.0325	0.325	171	174.784
23	0.0322	0.1625	0.0775	0.775	334	331.687
24	0.058	0.5	0.055	0.55	206	191.452
25	0.0326	0.3875	0.0775	0.325	149	152.83
26	0.012	0.275	0.055	0.55	182	195.591
27	0.056	0.275	0.055	0.55	208	196.135
28	0.0776	0.1625	0.0325	0.775	246	242.568
29	0.0323	0.1625	0.0325	0.775	293	294.424
30	0.0329	0.3875	0.0775	0.775	308	309.109

Table06: An ANOVA for quadratic models:

Source	Sum of Squares	Df	Mean Square	F-value	p-value	
Model	1.324E+05	14	9456.86	81.72	< 0.0001	Significant
A-Glucose	3725.05	1	3725.03	32.19	0.0011	
B-Yeast Extract	165.39	1	165.37	1.46	0.0025	
C-K_2HPO_4	900.36	1	900.33	7.77	0.0138	
D-Feather Meal	96901.03	1	96901.05	837.26	< 0.0001	
AB	248.08	1	248.07	2.16	0.1638	
AC	663.07	1	663.08	5.74	0.0302	
AD	6847.55	1	6847.57	59.17	< 0.0001	
BC	3052.54	1	3052.56	26.38	0.0001	
BD	33.09	1	33.07	0.2858	0.6008	
CD	1072.52	1	1072.58	9.29	0.0082	
A^2	1815.38	1	1815.33	15.66	0.0013	
B^2	122.66	1	122.63	1.04	0.3196	
C^2	3620.87	1	3620.85	31.29	< 0.0001	
D^2	12519.64	1	12519.68	108.18	< 0.0001	
Residual	1736.09	15	115.75			
Lack of Fit	1433.26	10	143.36	2.36	0.1771	not significant
Pure Error	302.88	5	60.54			
Cor Total	1.341E+03	29				



(A)



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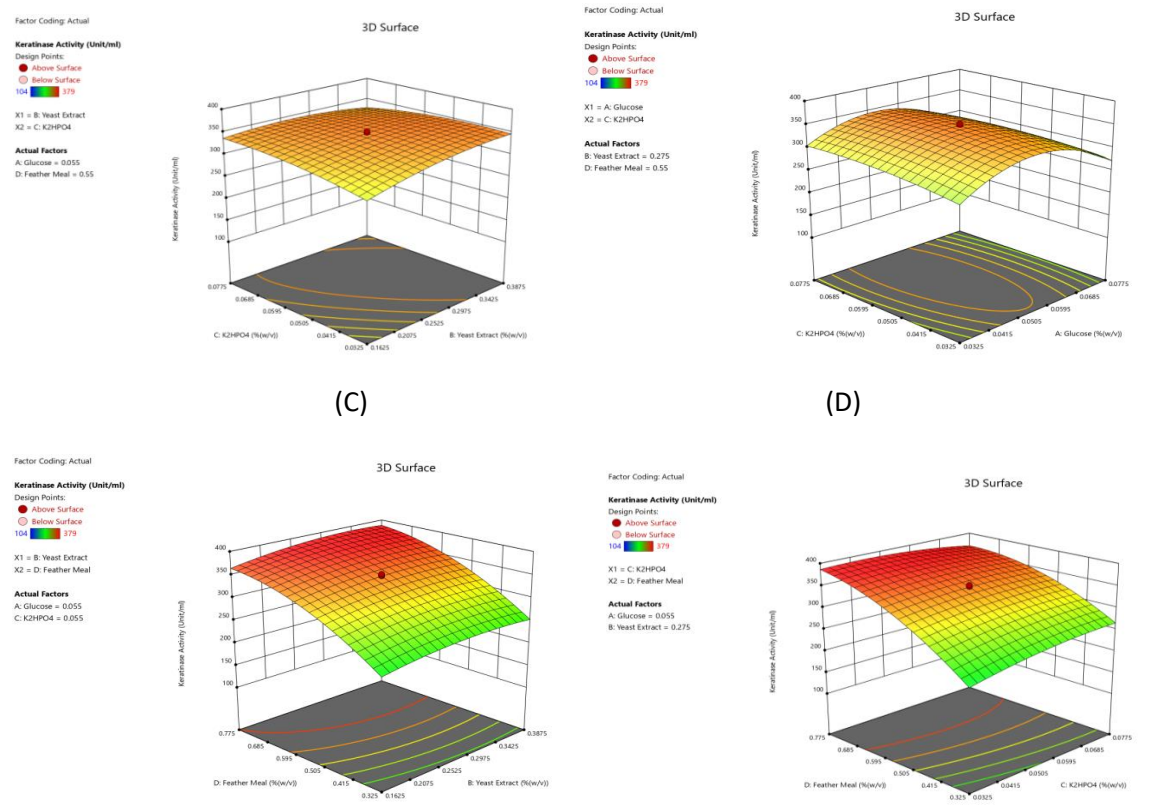

Figure 03: "An interaction between two parameters reveals the activity of keratinase in three dimensions."

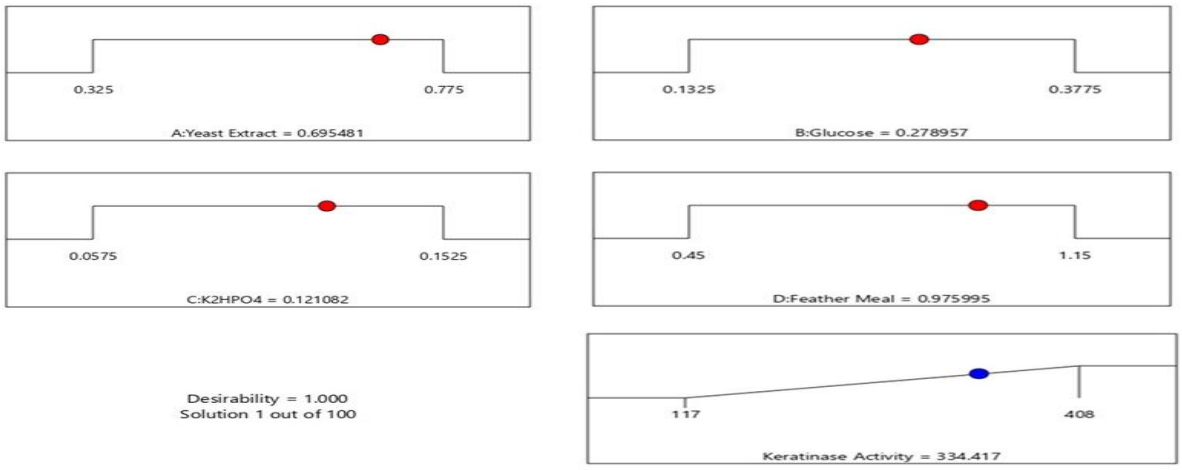

Figure 04: An optimal solution is represented by a desirability plot.

Ethical Approval:
No conflicts of interest to declare.
This research did not contain any studies involving animal or human participants, nor did it take place on any private or protected areas.

Consent to Participate:
The research may not be of directed benefit to us, participation is completely voluntary.

Consent to Publish:
Written informed consent for publication of their details was obtained from the study participants of kin.

Authors Contributions:
All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed Dr. Shailesh B. Patil and Prof. Jitendra D. More. All authors read and approved the final manuscript.

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Competing interests:
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