https://doi.org/ 10.33472/AFJBS.6.9.2024.38-57

African Journal of Biological Sciences

ENHANCING THE PRODUCTION OF KERATINASE BY *BACILLUS SUBTILIS* RAS04 BY STEP-BY-STEP OPTIMIZATION OF NUTRITIONAL PARAMETERS IN BIOPROCESSES.

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Article History

Volume 6,Issue 9, 2024

Received:14 Mar 2024

Accepted : 30 Mar 2024

doi: 10.33472/AFJBS.6.9.2024.38-57

ABSTRACT:

To enhance the productivity of ***Bacillus subtilis*** **RAS04**, a potent keratinase producer, for industrial purposes, it is important to improve the medium. RAS04 was identified using 16S rRNA sequencing and phylogenetic analysis. Additionally, the sequenced data was uploaded to the NCBI gene bank. By changing bioprocess nutritional factors, the use of PBD (Plackett-Burman Design) and CCD-RSM (Central Composite Design-Response Surface Methodology) is a step toward making optimized keratinase production on a large scale with *Bacillus subtilis* RAS04 (OQ519653.1). A qualitative optimization of eight nutritional bioprocesses was conducted using the PBD approach. Yeast extract, glucose, feather meal, and K_2HPO_4 significantly influence the variables. Quantitative optimization employing CCD-RSM has optimized the significant quantity of variables involved in keratinase production. Throughout a 72-hour incubation period in a modified optimized production medium, the sample exhibited favourable results. This was achieved by incorporating specific quantities of glucose, yeast extract, feather meal, and K_2HPO_4. The keratinase enzyme activity was measured at 334.41 units/ml, demonstrating a significant 2.38-fold increase in activity by using *Bacillus subtilis* RAS04 (OQ519653.1). In this study, feather meal and K_2HPO_4 were found to increase the keratinase synthesis of the common genus Bacillus. Typically, only carbon and nitrogen components are affected.

Keywords: Keratinase, screening, Optimization, Placket- Burkman design, central composite design-RSM.

1. Introduction:

The rising demand for food due to the growing human population has increased the production of keratinous wastes like feathers, wool, hooves, horns, etc. from poultry, animal husbandry, and slaughterhouses.[1] The continued presence of non-biodegradable and tough forms of keratinous waste presents a substantial risk to the sensitive equilibrium of the natural world. [2] Avian feather wastes are produced on a massive scale globally, making them one of the most prominent keratinous wastes. [3]In chickens, feathers make up approximately 5–7% of the total weight of the bird. [2] Feathers consist primarily of pure keratin (β-keratin), which is a rich source of amino acids, proteins, and nitrogen [4,5]. Kaewsalud et al. (2021) conducted a recent study that revealed the sample contained 1% [1]fat and 8% water. Keratin is known as a highly resistant protein that has gained recognition for its sustainability, despite the challenges presented in terms of recycling.[6] They are protein molecules with fibrous structures consisting of α-helix and β-sheet configurations and can be categorized into two types: soft keratins and hard keratins. Feathers contain hard keratins that possess sulfur, cysteine, and disulfide links, which contribute to their remarkable strength and stability.[6,7] The composition of amino acids in keratins can vary depending on their source. However, the overall structure of keratins predominantly consists of cysteine, glycine, serine, alanine, and valine, with smaller amounts of lysine, methionine, and tryptophan. The firm nature of keratins allows them to withstand the effects of hydrolytic enzymes like proteases (such as papain, trypsin, and pepsin) as well as various chemical treatments (including acidic, alkaline, and reducing agents).[3,8] This situation leads to the disposal of waste into the environment, either through landfills, which can have harmful effects on existing organisms, or through the combustion of materials, resulting in significant toxic pollution or incineration. [1,5] Studies have demonstrated the efficient breakdown of the main barrier in keratin-rich waste by a particular class of enzymes known as keratinases. [8] Microbial keratinases are becoming more widely understood and employed in the field of bioengineering due to their ability to effectively utilize resistant keratin sources and contribute to the creation of sustainable and environmentally friendly environments. [2]

Keratinases are a potential biocatalyst that can be used to degrade keratin-based sources. Various microbes secrete them, with bacterial keratinases being the most extensively produced. The degradation of keratin waste generates cleaner environments and produces value-added metabolites with various applications. Keratin hydrolysates have bioactive attributes, leading to their use in healthcare, also have antimicrobial, antioxidant, and angiotensin I-converting enzyme inhibitory activities. Other uses include thermo-bioplastics, compostable films, detergents, and nail treatments. Further research in isolating potent strains and characterization of keratinase is necessary to increase its application. To be able to achieve considerable yields of enzymes, the implementation of a suitable cultivation medium is required. As a result, the bioprocessing industry implements a variety of approaches, such as the traditional one-factor-at-a-time approach, and statistical methods such as response surface optimization design and Plackett Burman [9].

Optimization is crucial for commercial goals, but it often lacks the interacting effects among variables. Response Surface Methodology (RSM) is a useful technique to overcome this

limitation. It is a collection of mathematical and statistical techniques used to improve and develop processes affecting multiple variables and is crucial in the development, design, and formation of new products and improves existing designs. At the same time, it eliminates the drawbacks of conventional methods and has proven helpful in optimizing metabolites' production. Second-order models like Box-Behnken, Plackett-Burman, and Central Composite designs are widely used in RSM. Implementing RSM in fermentation processes can lead to increased product yields, reduced process variability, and reduced overall cost. However, studies often disregard these limitations[10].

The study aimed to enhance the production of alkaline protease in *Bacillus subtilis RAS04* by optimizing different variables in the production media. This was achieved using statistical techniques such as PBD and RSM through CCD.

2. Methodology

2.1 Materials

A variety of reagents, including substrates that provided nutrition and chemicals, were prepared by Merck from Mumbai, India; SD Fine from Baroda, Gujarat, India; Sigma Aldrich from St. Louis, Missouri, USA; and SRL from East Mumbai, India. HI Media (Mumbai, India) has provided a complete collection of laboratory media for use in the laboratory.

2.2 Microorganism

After conducting a thorough screening of over thirteen different soil samples containing keratin waste, ***Bacillus subtilis*** **RAS04 (OQ519653.1)** was discovered in the soil. Based on the testing results, this particular isolate was identified as the top producer of keratinase.

2.3 Conditions for cultivating and obtaining crude enzymes

The experiment was conducted using *Bacillus subtilis* RAS04 culture in submerged fermentation. The modified production medium consists of feathers 0.5 gm [11] and one set only containing 0.5gm feathers and salt medium (Carbon and nitrogen derived solely from feathers). Fermentation took place at 37ºC at 120 rpm for 48 hours on a sterile–modified production medium and basic salt medium inoculated with 3% active bacterial culture. As soon as the fermentation was completed, the supernatant was centrifuged at 10,000 g for 10 minutes at 4°C for analysis of Keratinase activity.

2.4 Keratinase assay:

During keratinase assay, amino acid (Tyrosine) released was determined using a modified Cai et al method. [12] After the reaction mixture had been added to the tubes, incubation in a water bath was performed until the reaction was completed at 50°C for 20 min. To stop the enzymatic reaction, 10% TCA was added. Adding an enzyme after the addition of TCA solution produced a blank that could be used. After precipitating at room temperature, it was centrifuged for 10 minutes at 4°C at 10000 g to remove the precipitated protein. The supernatant was collected separately in test tubes and absorbance at 280nm was measured using UV – Visible spectrophotometer. The below-mentioned formula was used to calculate Keratinase Activity by using the following inputs:

$$\boldsymbol{KeratinaseActivity(U/ml) = 4xNxA_{280}/0.01/20}$$

Were, N - dilution rate,
4 - final reaction volume,
20 - incubation time

In the definition of international units (IUs), a unit keratinase is defined as the amount of enzyme that can liberate one mol of amino acids per minute per millilitre (mol/mL/min).

2.5 Plackett – Burman design: screening of variable important for Keratinase production:

The purpose of this study is to determine the conditions that influence the maximum production of keratinase, Plackett – Burman design was employed for *Bacillus subtilis* RAS04. [13] According to the literature review, 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 was conducted to select significant variables, seven selected variables and four dummy variables, each of which had two levels (one high level and the other low level) as shown in **Table 1**. In order to maximize keratinase production, we optimized variables with 95% confidence level or higher using the Response Surface Methodology (CCD).

Table 1. 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

2.6 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, least lower (-2), lower (-1), middle (0), higher (+1), and higher (+2) with a thirty-run experiment as shown in **Table 2**. Six repetitions of the centre point are made to study the lack of fit and pure error of this model is undertaken. The equation involving polynomials of second order was given to explain the model's behaviour:

$$Y = \beta_0 + \sum_i \beta_i X_i + \sum_{ii} \beta_{ii} X_i^2 + \sum_{ij} \beta_{ij} X_i X_j$$

As a point of reference, it should be noted that in this case, Y represents the predicted outcome (Keratinase activity), β_0 is the model constant, β_i represents the linear coefficient, β_{ii} represents the quadratic coefficient, and β_{ij} represents the interaction coefficient. The independent coded variables are represented by X_i and X_j, respectively. [14]

Using Design-Expert software, a regression analysis was conducted. A model with a large degree of fit and a p-value lower than 0.05 was compared with a model with an insignificant lack of Fit test. The statistical significance of the proposed model equation was evaluated using the fit statistics values 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 of adjusted and predicted value. Additionally, diagnosis and influences of different types of plot results were 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. To assess the production of keratinase, Plackett-Burman experiments were conducted using independent two-level variables.[15]

Table 2. RSM independent variable ranges

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

3. Results and Discussion:

3.1 Plackett–Burman design

Twelve experiments with four dummy variables were conducted using the PBD (Plackett-Burman design) to examine seven parameters of the process and significant variables were determined for keratinase production. According to **Table 3**, the experimental design and response (Keratinase activity) are shown as below. Among the variables selected for the process, glucose, yeast extract, feather meal, and K_2HPO_4 are positively related to Keratinase production (Table 4), while corn flour, $CaCl_2$, and pH is negatively related. In **Figure 1**, the Pareto chart shows the order of significance of these variables with orange factors (dark colour bars) showing a positive effect and blue factors (light colour bars) showing a negative effect on Keratinase production. As depicted in **Figure 2**, glucose, yeast extract, feather meal, and K_2HPO_4 have a significantly greater effect on Keratinase production than other factors. Normal distributions are observed for all other factors along a straight line, despite these four factors falling below or to the right. The reason is that they do not affect Keratinase production as much. Based on the results of the ANOVA, the Model F-value is 28.31, meaning that the model is significant. In the absence of noise, there is only a 0.30% chance that such a large F-value could be obtained. This is evident from the fact that terms used in model A, B, F, and H, namely Glucose, Yeast extract, Feather meal, and K_2HPO_4, are significant terms with p-values of 0.05 or lower (95% confidence interval). Keratinase production by *Bacillus subtilis* RAS04 is significantly influenced by these four nutritional parameters (**Table 4**).

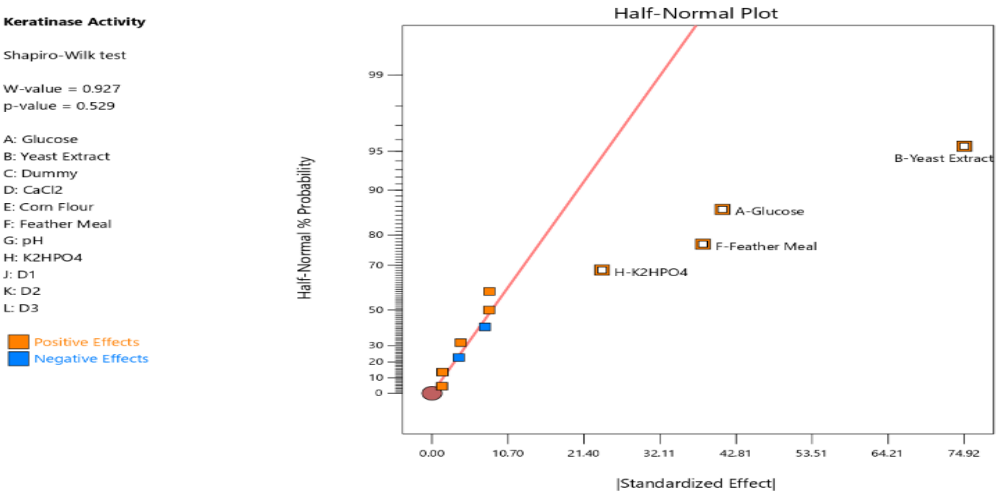
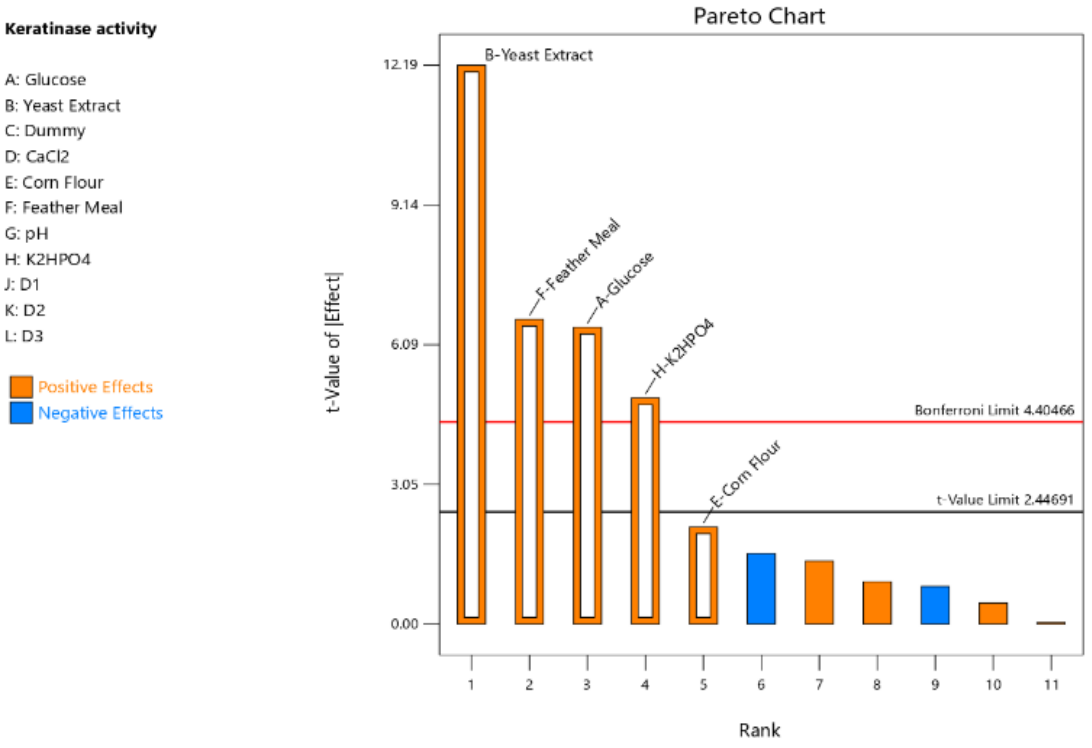


Figure 1. An outlier plot shows a significant effect on keratinase production when compared to a half-normal plot.

Figure 2. Pareto chart of 11 factors showing order of significance.



3.2 CCD-RSM optimization of selected variables to maximize Keratinase production

A central composite design of response surface methodology was used to optimize the concentration of each variable based on the results of the Plackett Burman design of the significant effect of process variables on Keratinase production. As shown in **Table 5**, Thirty several experimental designs were run in CCD-RSM, and a set of experimental and predicted responses for each numeric factor at various levels (rotatable with 6 replications at the center point) is presented. As a result of the model statistics' F-value (81.71) being low, a quadratic model was recommended by Design-Expert software for the model. In this case, noise is only responsible for 0.01% of the F-value. Determination coefficients measure a number's significance and the model's adaptability, which is approximately near 1 (R^2=0.9871), measuring and predicting responses are more closely correlated. Additionally, the adjusted R^2 of 0.9750 is within reasonable limits of the predicted R^2 of 0.9352. This study requires a model with a signal-to-noise ratio of 38.0287, which is sufficient for providing sufficient precision. According to the lack of fit F-value of 2.37, the gap between the model and the pure error is not significant (for the model to fit, the gap between the model and the pure error must be non-significant). Noise is responsible for 17.70% of the time for a large lack of Fit F values as shown in **Table 6**. According to these fit statistics, the quadratic model suggested that higher Keratinase production is appropriate. Design-Expert software was used to verify the appropriateness of the recommended model using diagnostics and influence tools. It shows a straight line if there is a normal probability; therefore, residuals are normally distributed. Plots of residuals versus predicted response values were investigated for checking constant variance.

As a result, it was not evident that variance had expanded from a lower predicted score to a higher predicted score in these plots. The residual versus run plot did not exhibit any kind of trend. An analysis of a box-cox plot was conducted to apply power transformations. Lambda values between the range of 0.3812 to 1.3864 in the 95% confidence interval, with 0.9210 being the most significant. A power transformation is not recommended because, within the range given, A lambda value has been suggested for the suggested model that is close to the best lambda value. It appears that the proposed model is adequate based on all of these diagnostics. To predict the response of each factor, this model is based on an equation using coded factors for a second-order polynomial. By comparing the coefficients of the factors, it can be used to identify their relative influence:

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

Table 3. 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 4. An analysis of the Plackett-Burman Design Experiment statistics

Source	Sum of Squares	Df	Mean Square	F-value	p-value	
Model	29636.71	7	4233.82	28.31	0.0030	Significant
A-Glucose	4700.52	1	4700.52	31.44	0.0050	
B-Yeast Extract	16673.11	1	16673.11	111.51	0.0005	
D-CaCl2	75.50	1	75.50	0.5049	0.5166	
E-Corn Flour	503.11	1	503.11	3.36	0.1405	
F-Feather Meal	4957.27	1	4957.27	33.15	0.0045	
G-pH	0.1408	1	0.1408	0.0009	0.9770	
H-K_2HPO_4	2727.07	1	2727.07	18.24	0.0129	
Residual	598.11	4	149.53			
Cor Total	30234.82	11				

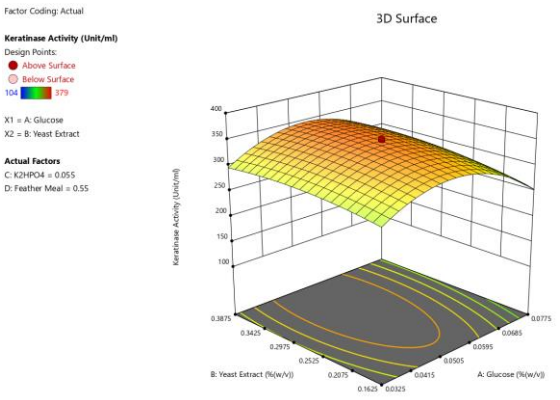
Table 5. Design layout for the CCD-RSM using four coded variables with experimental and predicted outcomes.

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	0.055	0.275	0.01	0.55	226	230.525
2	0.0325	0.1625	0.0775	0.325	203	190.225
3	0.0775	0.3875	0.0775	0.775	235	237.05
4	0.055	0.275	0.1	0.55	244	256.092
5	0.0775	0.3875	0.0775	0.325	179	163.092
6	0.055	0.275	0.055	0.55	194	196.1
7	0.055	0.05	0.055	0.55	190	192.258
8	0.0775	0.1625	0.0775	0.325	169	187.317
9	0.0325	0.3875	0.0325	0.325	138	131.092
10	0.1	0.275	0.055	0.55	132	154.025
11	0.055	0.275	0.055	0.55	191	196.1
12	0.0325	0.1625	0.0325	0.325	117	122.517
13	0.055	0.275	0.055	0.55	183	196.1
14	0.0325	0.3875	0.0325	0.775	338	317.85
15	0.055	0.275	0.055	1	408	396.242
16	0.0775	0.1625	0.0325	0.325	147	153.008
17	0.055	0.275	0.055	0.1	141	150.375
18	0.055	0.275	0.055	0.55	195	196.1
19	0.0775	0.1625	0.0775	0.775	255	246.425
20	0.055	0.275	0.055	0.55	188	196.1
21	0.0775	0.3875	0.0325	0.775	270	279.192
22	0.0775	0.3875	0.0325	0.325	176	174.783

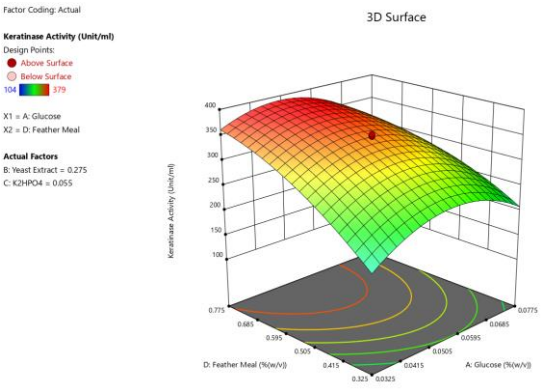
23	0.0325	0.1625	0.0775	0.775	334	331.683
24	0.055	0.5	0.055	0.55	205	191.458
25	0.0325	0.3875	0.0775	0.325	148	152.8
26	0.01	0.275	0.055	0.55	181	195.592
27	0.055	0.275	0.055	0.55	206	196.1
28	0.0775	0.1625	0.0325	0.775	241	242.567
29	0.0325	0.1625	0.0325	0.775	290	294.425
30	0.0325	0.3875	0.0775	0.775	305	309.108

Table 6. An ANOVA for quadratic models.

Source	Sum of Squares	Df	Mean Square	F-value	p-value	
Model	1.324E+05	14	9456.87	81.71	< 0.0001	Significant
A-Glucose	3725.04	1	3725.04	32.18	0.0011	
B-Yeast Extract	165.38	1	165.38	1.43	0.0025	
C-K_2HPO_4	900.38	1	900.38	7.78	0.0138	
D-Feather Meal	96901.04	1	96901.04	837.24	< 0.0001	
AB	248.06	1	248.06	2.14	0.1638	
AC	663.06	1	663.06	5.73	0.0302	
AD	6847.56	1	6847.56	59.16	< 0.0001	
BC	3052.56	1	3052.56	26.37	0.0001	
BD	33.06	1	33.06	0.2857	0.6008	
CD	1072.56	1	1072.56	9.27	0.0082	
A^2	1815.36	1	1815.36	15.68	0.0013	
B^2	122.65	1	122.65	1.06	0.3196	
C^2	3620.86	1	3620.86	31.28	< 0.0001	
D^2	12519.65	1	12519.65	108.17	< 0.0001	
Residual	1736.08	15	115.74			
Lack of Fit	1433.25	10	143.32	2.37	0.1770	not significant
Pure Error	302.83	5	60.57			
Cor Total	1.341E+05	29				



(A)



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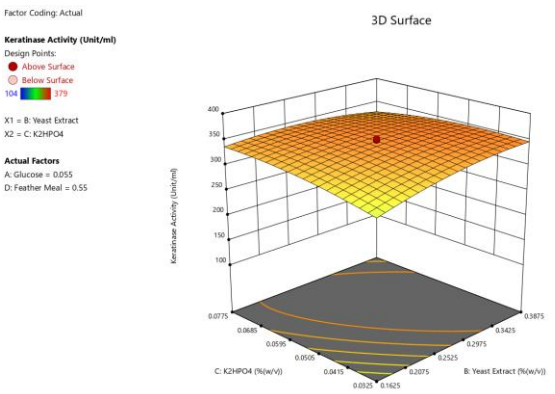

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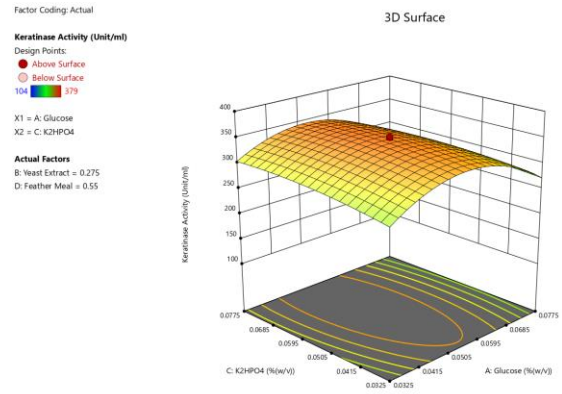

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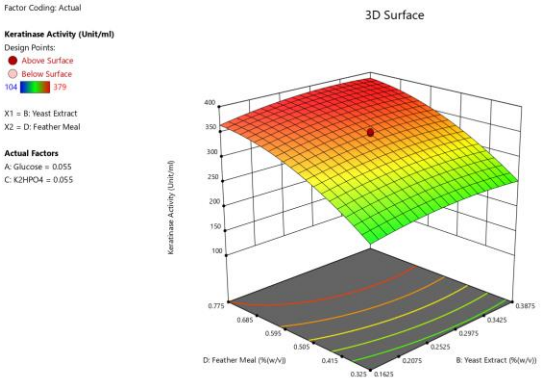

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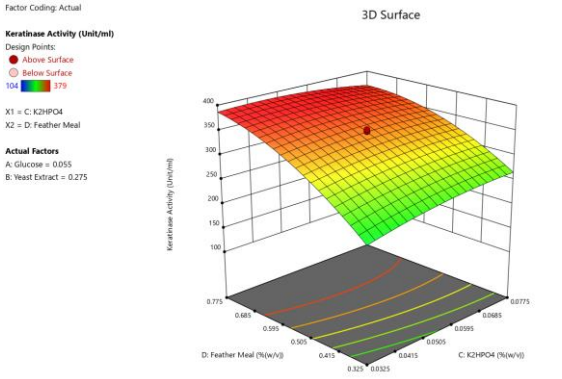

(F)

Figure 3. An interaction between two parameters reveals the activity of keratinase in three dimensions.

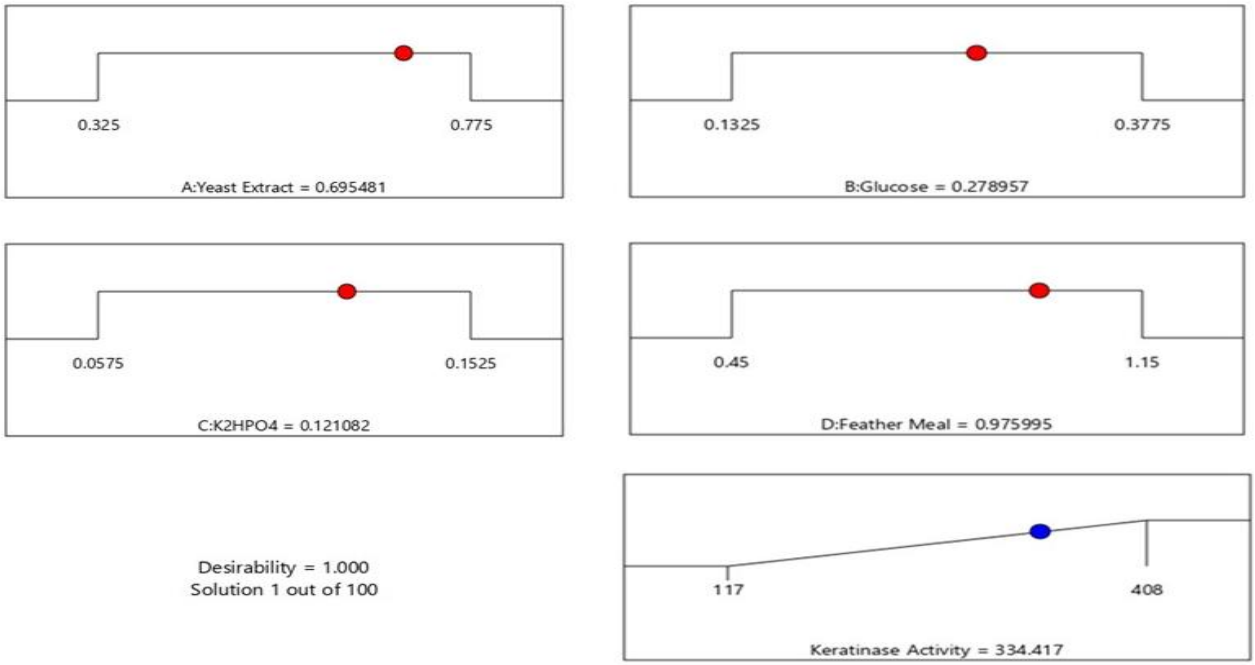

Figure 4. An optimal solution is represented by a desirability plot.

The response was plotted against any combination of two numerical factors and all other parameters were maintained at their mid-values to study the interaction effect of selected process variables as shown in **Figure 3**. The maximum Keratinase production is observed when Yeast Extract is higher and glucose is in the midrange mentioned in **Figure 3A**. As with Feather Meal, a low glucose amount and a high Feather Meal value make Keratinase production optimal as observed in **Figure 3B**. The concentration of K_2HPO_4 and yeast extract can be increased to increase Keratinase production as mentioned in **Figure 3C**. According to **Figure 3D**, maximum Keratinase production takes place at glucose levels in the middle and K_2HPO_4 levels in the higher ranges. A combination of yeast extract and feather meal giving maximum Keratinase production can be found in **Figure 3E** at the lowest values and the highest values at the higher ranges. Increased Feather Meal and decreased K_2HPO_4 concentration can be observed to increase Keratinase production mentioned in **Figure 3F**. It is necessary to use an abundance of feather meals to maximize keratinase production, but K_2HPO_4 or yeast extract is needed if the feather meal is mid-value. Over a certain range of glucose levels, keratinase production does not significantly change.

According to Design-Expert software, the optimal solution is shown in the desirability plot found in **Figure 4**. To maximize Keratinase production, glucose should be added at 0.278%, K_2HPO_4 at 0.121%, feather meal at 0.975%, yeast extract at 0.695% with pH 9.00,

and incubation time at 72 hours using *Bacillus subtilis* RAS04. A laboratory flask experiment was conducted in triplicate to validate the suggested optimized solution using *Bacillus subtilis* RAS04 in the modified feather basal medium. Predicted response data were compared with results from validation experiments. A 95% prediction interval range of Keratinase production was observed in the experimental response data (334.41 Units/ml), which was within the experimental guidelines for prediction (117-408 Units/ml). Response surface methodology's central composite design tool resulted in higher Keratinase production than the basal medium (approximately 2.38-fold).

The use of *Bacillus* species has recently been reported. CCD design applied to *Bacillus sp*. UPM-AAG1 optimized keratinase medium results in a 1.7-fold increase in keratinase yield. [15]As a comparison, CCD-RSM optimization using *Bacillus pumilus* AI combined with response surface methodology led to around 87.73 units/ml of Keratinase production compared to basal media increment of 3.40-fold achieved production [16]. Various Bacillus species and their comparative studies regarding the same reported work are noted in **Table 7,** along with the substrate used for optimization and its production and fold.

Glucose, a crucial medium component, influences keratinase production. In some species, high glucose concentrations increase keratinase production, whereas higher concentrations decrease due to catabolite repression. This study demonstrates that keratinase production reaches its peak at glucose concentrations of 0.278%, and declines at concentrations exceeding this threshold [16, 17, 18, 19].

Keratin biodegradation using inorganic or organic nitrogen sources has a significant impact. Yeast extract, a key component in keratinase production, can increase keratinase production in some species but reduce it in others. Optimizing yeast extract concentrations for different species is crucial for optimal enzyme production. Previous research shows yeast extract-supplemented media yield the highest levels of keratinase [16, 17,18].

The study found that keratinase production is inducible using a feather substrate, which is the best source for enzyme production. The optimal concentration of feather meal is crucial for enzyme growth and production. The study also found that higher feather meal concentrations do not significantly affect keratinase production. Higher concentrations can suppress catabolic activity, resulting in reduced enzyme production. Researchers reported similar work on *Bacillus sp. UPM-AAG1* and *Bacillus velezensis NCIM 5802* [9,15].

Inorganic salts like nitrogen and phosphorus, which vary depending on the microbial strain, influence Keratin biodegradation. K_2HPO_4 is a crucial component in the medium for keratinase production, with concentrations affecting enzyme production differently. Concentrations of K_2HPO_4 above 0.121% increase keratinase production, whereas concentrations below 0.121% decrease it. Inorganic phosphorus can be an initial source of phosphate during the early fermentation stages. In *Bacillus cereus L10* and *Bacillus paramycoides*, K_2HPO_4 stimulates keratinase production [3,17]

Table 7. Descriptive Summary of keratinase production by different Bacillus species.

Organism	**Optimization method**	**Substrates used for optimization**	**Keratinase Activity**	**Fold**	**References**
Bacillus sp. UPM-AAG1	PBD CCD-RSM	Feather Meal	60.1 U/mL	1.7	[15]
Bacillus cereus N14	OFAT CCD-RSM	Maltose yeast extract	74.86 U/mL	2.75	[16]
Bacillus cereus	OFAT RSM	Wheat bran Lactose	292 U/gm	----	[17]
Bacillus halotolerans L2EN1	OFAT CCD-RSM	Sucrose Yeast extract	65.50 U/ml	1.68	[18]
Bacillus subtilis AMR	CCD-RSM	Sucrose Yeast extract	418.70 U/ml	1.5	[19]
Bacillus paramycoides	PBD RCCD	Feather Meal K_2HPO_4	263.20 U/gm	-----	[20]
Streptomyces swerraensis KN23	CCD-RSM	Sucrose Yeast extract	129.60 U/ml	------	[21]
Bacillus velezensis NCIM 5802	RSM	Feather Meal	109.70 U/ml	4.92	[22]
Bacillus cereus L10	PBD	Yeast extract K_2HPO_4	9602 U/ml	4.56	[23]
Bacillus subtilis ES5	CCD-RSM	β-mercaptoethanol and EDTA	132.56 U/ml	2	[24]
Bacillus subtilis RAS04	PBD CCD-RSM	Glucose, Feather Meal K_2HPO_4	334.41 U/ml	2.38	

4.0 Conclusion

This study's findings highlight the impact of ***Bacillus subtilis RAS04*** on keratinase production. In previous studies, researchers usually used sucrose as the carbon source. However, this particular study observed that the introduction of a small quantity of glucose and yeast extract had a notable impact on the production process. By using an enhanced medium for laboratory-scale production and thoroughly characterizing these enzymes, we can demonstrate their potential applications in treating prion diseases, improving animal feed, accelerating leather industry processes, and enhancing agricultural techniques.

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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 information consent for publication of the 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 by Mr. Rishit Atulbhai Soni and Dr. Aarti V Thakker. All authors read and approved the final manuscript.

Funding:

The authors declare that no funds, grants, or other support were received during the preparation of this manuscript.

Competing interests:

The authors have no relevant financial or non-financial interests to disclose.