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Enhanced Production of Keratinase enzyme from Chicken feathers using Response Surface Methodology

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Abstract: Medium composition for production of Keratinase by *Bacillus subtilis (NCIM No-2724*) using powdered chicken feather as substrate in submerged fermentation was optimized using response surface methodology. Plackett-Burman design was employed for screening the most significant nutrient components which influence the Keratinase production from eleven nutrient components. The effect of eleven nutrient supplements in the SmF medium were analysed using Plackett-Burman design and the nutrients namely Peptone, NaCl, KCl and K_2HPO_4 were found to be most significant for the production of Keratinase with confidence levels greater than 95%. Central composite design (CCD) was used to determine the optimal concentrations of these four most significant components to obtain the maximum enzyme yield and the experimental results were fitted with a second-order polynomial model. The optimum conditions are: peptone (0.53939%w/v), NaCl (0.09485%w/v), KCl (0.09485%w/v) and K_2HPO_4(0.13384%w/v). Under the above optimized conditions the maximum Keratinase activity was found to be 92.28 IU/ml, with the R^2 value of 0.9988 indicates the fitness of the model to predict the experimental data.
Keywords : Keratinase, central composite design, *Bacillus subtilis*, Powdered Chicken feather substrate, Plackett-Burman design.

1. Introduction

Microbial keratinases (EC 3.4.21/24/99.11) are amongst the industrially important enzymes. They are proteolyticenzymes capable of hydrolyzing highly rigid keratin which is recalcitrant to commonly known proteases such as trypsin, pepsin and papain [1]. They are finding applications in leather and detergent industries, textile, waste bioconversion, medicine and cosmetics for drug delivery through nails and degradation of keratinizedskin[2].Keratinases have applications in traditional industrial sectors including feed, detergent, medicine, cosmetics and leather manufacturers [3], they also find application in more recent fields such as prion degradation for treatment of the dreaded mad cow disease [4], biodegradable plastic manufacture and feather meal production and thus can be appropriately called “modern proteases”. The use of keratinases to enhance drug delivery in some tissues and hydrolysis of prion proteins arise as novel potentially high impact applications for these enzymes [5]. Although many applications of keratinases are still in the stage of infancy, a few have found their way to commercialization, particularly the use of Bioresource International’s (BRI) Versazyme for feather meal production. The crude enzyme can also serve as a nutraceutical product, leading to significant improvement in broiler performance [6]. The most promising application of keratinase is in the production of nutritious, cost effective and environmentally benign feather meal [7]. Nutritional enhancement can be achieved by hydrolysis of feather meal/raw feather using keratinase which significantly increases the levels of essential amino acids methionine, lysine and arginine [8].

Biodegradation of feather keratin by microorganisms producing keratinases represents an alternative

method to improve the nutritional value of feather waste and to prevent environmental contamination. Environmental wastes are found in large quantities in many countries. Although some of them contain a considerable amount of protein and various carbon compounds, little attention is given to utilizing or recycling this waste in a technological way. Additionally, the accumulation of some of these wastes in nature is considered to be a serious source of pollution and health hazards. Therefore, their proper disposal may be considered as a means of avoiding environmental pollution. Recent works focused on the utilization of some polymeric wastes, mainly feather wastes. Feathers are generated in large amounts as a waste by product at commercial poultry-processing plants reaching millions of tons per year worldwide [9].

Microbial keratinases are promising biocatalysts for several purposes, including applications in fertilizer, feed, detergent, leather, textile industries, and also for biomedical and pharmaceutical applications. At present, the applications are aimed at environmental friendly activities. However microbial keratinases are considered as promising biocatalysts for several pharmaceutical and biomedical applications. Bioconversion of Keratin-rich materials into amino acids, peptides and soluble proteins by keratinaseis possible[10].Keratinases have recently gained biotechnological impetus because of their ability to act on hard-to-degrade proteins such as hair, feather, nail, etc and thus, becoming a part of solid waste management as recycling of these wastes is tough[11].Because of environmental considerations the use of keratinolytic enzymes in the production of amino acids and peptides is becoming attractive for biotechnological applications. Due to insoluble nature of keratin, it isresistance to enzymatic digestion by plants, animals and many known microbial proteases. Therefore the keratinase producing microorganisms have been described having the ability to degrade feather.

A number of keratinolytic microorganisms have been reported, including some species of fungi such as *Microsporum* [11], *Trichophyton*[10] and from the bacteria *Bacillus* [12], *Streptomyces*[1] and actinomycetes [14]**.** Keratinases have enormous potential applications in processing waste in the poultry and leather industries. Moreover, feather waste represents a potential protein alternative to more expensive dietary ingredients for animal field [15**].** Bacterial keratinases are of particular interest because of their action on insoluble keratin substrates, andgenerally on a broad range of protein substrates [16].Several bacteria produce keratinase as an extracellular material. Most of these belong to the genus *Bacillus*. These bacteria use keratinous substrates such as chicken feathers as carbon sources for the production of keratinase. The production of keratinase is usually most noticeable when chicken feathers are used as a sole carbon source.

Cultivation conditions are essential in successful production of an enzyme, and optimization of media composition is important in developing the cultivation process. Submerged fermentation of poultry waste by microorganism producing keratinase helps in the conversion of non-soluble keratin (feather) into soluble protein or polypeptide [17].For a broad application, the cost of bio-products is one of the main factors determining the economics of a process. Reducing the costs of enzyme production by optimizing the fermentation medium is the basic research for industrial application. The use of different statistical designs for medium optimization has been recently employed for many enzymes, antibiotics, and metabolites. Response surface methodology is an empirical statistical modeling technique employed for multiple regression analysis using quantitative data obtained from properly designed experiments to solve multivariable equations simultaneously [18].

The present work was, therefore, planned for the response surface methodological optimization of medium composition for enhanced keratinase production by *Bacillus subtilis No- 2724* under submerged fermentation utilizing powdered chicken feather as a sole carbon source. The medium optimization was carried out by three steps (1) screening the most significant components influencing Keratinase production by using Plackett-Burman design (2) Optimization of the most significant medium components by applying central composite design (CCD) of Response Surface Methodology (RSM) and (3) Experimental validation of the model by carrying out the batch experiments in the laboratory under the optimized condition.

2. Materials and Methods

2.1 Microorganism and maintenance

Bacillus subtilisNo- 2724 used in this study was purchased from NCIM Resource National Chemical Laboratory, Pune-Maharashtra.

The medium used for the growth and maintenance of the culture comprises of (g /L):Sodium chloride (NaCl) - 0.5, Ammonium Chloride (NH_4Cl) - 0.5, Di-potassium hydrogen orthophosphate (K_2HPO_4) - 0.3, Potassium hydrogen phosphate (K_2HPO_4)- 0.3, Magnesium Sulphate ($MgSO_4$) - 0.1,Yeast extract - 0.1,Distilled water - 1000 ml(pH 7.5 ± 0.2),

Bacterial cells in agar slants were incubated at 37°C for 24 h and the stock culture was stored at 4°C.

For the production of keratinase, the medium comprises of, (g/L): Raw feather, 10;NH_4Cl, 0.5; NaCl, 0.5; K_2HPO_4, 4.0; KH_2PO_4, 0.3 was used. Initial pH of the medium was adjusted to 7.5 ± 0.2 with Tris–HCl buffer. The medium was sterilized in an autoclave for 15 min at 121°C[15].

2.2 Preparation of Raw feather broth

1000ml of raw feather broth were prepared and autoclaved at 121°C for 15minutes. The sterile pre-weighed feather pieces were aseptically transferred into respective broth. A loopful of bacterial culture *Bacillus subtilis* was inoculated into respective medium. 250ml Erlenmeyer flask containing only the feather was maintained as control. These flasks were incubated at 37°C for 5days. The Culture supernatants obtained after centrifugation at 8000×*g* for 20 min were used for further study.

2.3 Substrate and Reagents

Chicken feathers were collected from the poultry shop in Cuddalore District. Chicken feathers were washed three times with distilled water, dried and ground. All chemicals used were of the AR grade and were purchased from Hi Media Limited, Mumbai, India.

2.4 Preparation of native chicken feather (Substrate)

Native chicken feathers were cut with scissors to small pieces of 0.5-1.0 cm long, and then washed several times with tap water. Defatted of feather pieces were done by soaking them in a mixture of chloroform: methanol (1:1 v/v) for 2 days followed by chloroform: acetone: methanol (4:1:3 v/v/v)for 2 days. The solvent was replaced every day. Finally, the feathers were washed several times with tap water to eliminate the solvent residual, dried for 3 days at 50°C and grinded using electrical mixer grinder. Grinded keratin which looked like cotton was maintained in a sterile bottle at 4°C for further use.[19]

2.5 Preparation of Keratin

The collected native feathers were prepared according to the method of [20]. Keratin was obtained from the prepared feathers by the modified method of [21] and used as a keratin powder.

2.6 Preparation of Keratin solution

Keratinolytic activity was measured with soluble keratin (0.5%, w/v) as substrate. Soluble keratin was prepared from white chicken feathers by the method of [21]. Native chicken feathers (10 g) in 500 ml of dimethyl sulfoxide were heated in a hot air oven at 100 °C for 2h. Soluble keratin was then precipitated by addition of cold acetone (1 L) at −70 °Cfor 2 h, followed by centrifugation at 10, 000×g for 10 min. The resulting precipitate was washed twice with distilled water and dried at 40°C in a vacuum dryer. One gram of quantified precipitate was dissolved in 20ml of 0.05M NaOH. The pH was adjusted to 7.0 with 0.1M hydrochloric acid and the solution was diluted to 200 ml with 0.05 mol/L Phosphate buffer (pH 7.0)[22].

2.7 Production of Keratinase enzyme:

2gof the powdered substrate was mixed with 100ml of the corresponding mineral salt media defined by the experimental design, in a 500ml Erlenmeyer flask. After adjusting the pH to 8, each flask was covered with hydrophobic cotton and autoclaved at 121^0C and 15 psi pressure for 15 minutes. Medium was inoculated with 4%(v/v) of inoculum after cooling it down to room temperature. The inoculated flasks were incubated at 37^0C in an orbital shaker incubator.

During the preliminary screening process, the experiments are carried out for 120 hours and it was found that at the 48 hr, the maximum production occurs. Hence all the experiments are carried out for a fermentation period of 48 hrs.

2.8 Extraction of Keratinase

At the end of the fermentation period, contents of the flask were filtered using a Whatman No.1 filter paper. The filtrate was then centrifuged at 10,000 rpm for 10 minutes and the supernatant was used as the crude enzyme to examine keratinase assay.

2.9 Keratinase assay

The keratinase activity was assayed as follows: 1ml of the enzyme solution was incubated with keratin powder (0.5% w/v) in 2ml of 0.1 M phosphate buffer, pH 7.0 for 1h at 40°C in a shaking water bath for 10 min.

The reaction was blocked by adding 2ml of 20% TCA and followed by filtration to eliminate the unutilised substrate. To 1ml of the filtrate 2.5 ml of 0.5N sodium bicarbonate solution and 0.5 ml of dilutedfolin phenol ciocalteau reagent were added. The reaction mixture was incubated for 30 min. The control was prepared by incubating the enzyme solution with 2.0 ml of TCA without the addition of substrate. TheFolin Ciocalteau reagent is used as colouring reagent and the absorbance was measured at 660nm. One unit of enzyme is defined as the amount of enzyme required to liberate 1 micro mole of tyrosine per min under optimal experimental conditions. [12]

2.10 Statistical Analysis

I Screening of medium components using Plackett-Burman design

Table 1: Nutrient screening using a Plackett-Burman design

		Levels	%(wt./vol.)
Nutrient code	**Nutrient**	**Low (-1)**	**High (+1)**
A	Yeast extract	0.01	0.5
B	$(NH_4)_2Cl$	0.02	0.8
C	KH_2PO_4	0.05	0.1
D	NaCl	0.02	0.1
E	KCl	0.01	0.5
F	$MgSO_4$	0.01	0.1
G	$Feso_4.7H_20$	0.01	0.4
H	Peptone	0.01	0.05
J	$NaNO_3$	0.05	0.2
K	Urea	0.01	0.1
L	K_2HPO_4	0.01	0.1

Table 2: Plackett–Burman experimental design matrix for screening of important variables for Keratinase production

Run No	A	B	C	D	E	F	G	H	J	K	L	Keratinase Activity (IU/ml)
1	0.1	0.8	0.01	0.2	0.5	0.1	0.01	0.01	0.5	0.1	0.01	63.12
2	0.01	0.8	0.2	0.01	0.5	0.1	0.4	0.01	0.5	0.01	0.1	43.00
3	0.1	0.2	0.2	0.2	0.5	0.01	0.4	0.05	0.5	0.01	0.01	18.00
4	0.01	0.8	0.01	0.2	0.01	0.1	0.4	0.05	1.0	0.01	0.01	20.00
5	0.1	0.2	0.2	0. 01	0.5	0.1	0.01	0.05	1.0	0.1	0.01	25.00
6	0.01	0.2	0.01	0.2	0.5	0.01	0.4	0.01	1.0	0.1	0.1	22.00
7	0.1	0.2	0. 01	0. 01	0.01	0.1	0.4	0.05	0.5	0.1	0.1	64.00
8	0.1	0.8	0. 01	0. 01	0.5	0.01	0.01	0.05	1.0	0.01	0.1	33.00
9	0.1	0.8	0.2	0. 01	0.01	0. 01	0.4	0.01	1.0	0.01	0.01	64.00
10	0.01	0.8	0.2	0.2	0.01	0. 01	0.01	0.05	0.5	0.1	0.1	54.00
11	0.1	0.2	0.2	0.2	0.01	0.1	0.01	0.01	1.0	0.01	0.1	32.00
12	0.01	0.2	0.01	0. 01	0.01	0.01	0.01	0.01	0.5	0.01	0.01	25.00

The first step of optimization strategy is to identify the medium components that have significant effects on Keratinase production. Placket-Burman design is an effective and efficient technique for the optimization of medium components and can be used to select the significant factors and to eliminate the insignificant one in order to obtain more manageable and smaller set of factors [23]. Based on the Plackett-Burman design, each factor is examined at two levels, low (−1) and high (+1). This design assumes that there are no interactions between the different media constituents, x_i in the range of variable under consideration. To determine the variable that significantly affect the Keratinase production, Plackett-Burman design using statistical software package MINITAB (Release 15.1, PA, USA), is used. For screening purpose a total of eleven medium components (Table 1) are tested for their significance in 12 experimental runs (Table 2) and insignificant ones are eliminated. Table 1 illustrates the factors under investigation as well as the levels of each factor used in the experimental design. Table 2shows the Plackett-Burman design matrix. Significant nutrient components viz. peptone, NaCl, KCland K_2HPO_4 which increases the Keratinase production was identified.

II Optimization using Central Composite Design of Response surface methodology

In order to enhance the production of Keratinase, central composite design of Response Surface Methodology (RSM) is employed to optimize the most significant factors, identified by the Plackett–Burman (PB) design. This method can eliminate the drawbacks of single factor optimization [24]. RSM is useful for small number of variables (up to five) but is impractical for large number of variables, due to high number of experimental runs required. CCD was used to obtain a quadratic model, consisting of factorial trails and star points to estimate quadratic effects and central points to estimate the pure process variability with Keratinase production. The effects of the most significant variables on Keratinase production are studied by central composite design using Response surface Methodology. In the present study the statistical model is obtained using the Central Composite Design (CCD) with four independent variables peptone, NaCl, KCl and K_2HPO_4. CCD contains a factorial matrix with a centre point and "axial points" around the centre point that allow the curvature of the model to be established

Table: 3 Ranges and Levels of the independent variables used in RSM for Keratinase enzyme production

Sl. No.	Variables	Code	Levels				
			-2	-1	0	+1	+2
1.	Peptone (%)	**A**	0.20	0.35	0. 50	0.65	0.80
2.	Nacl (%)	**B**	0.01	0.06	0.11	0.16	0.21
3.	Kcl (%)	**C**	0.01	0.06	0.11	0.16	0.21
4.	K_2HPO_4 (%)	**D**	0.05	0.09	0.13	0.17	0.21

Table: 4 Experimental conditions (coded values) and observed response values of 2^4 central composite design for Keratinase enzyme production

Run No	Independent Variables								Keratinase Activity(IU/ml)	
	Orthogonal Values				Real Values					
	A	B	C	D	Peptone (%)	Nacl (%)	Kcl (%)	K_2HPO_4 (%)	Exp.	Pred.
1	0	0	0	0	0.50	0.11	0.11	0.13	92.00	92.00
2	-1	1	-1	-1	0.35	0.16	0.06	0.09	44.91	45.43
3	0	0	0	0	0.50	0.11	0.11	0.13	92.00	92.00
4	-2	0	0	0	0.20	0.11	0.11	0.13	48.58	48.63
5	0	-2	0	0	0.50	0.01	0.11	0.13	62.17	62.21
6	0	0	2	0	0.50	0.11	0.21	0.13	53.57	54.04
7	1	1	1	-1	0.65	0.16	0.16	0.09	61.05	61.17
8	0	0	0	0	0.50	0.11	0.11	0.13	92.00	92.00
9	0	0	0	0	0.50	0.11	0.11	0.13	92.00	92.00
10	0	0	0	2	0.50	0.11	0.11	0.21	65.33	66.03
11	1	1	1	1	0.65	0.16	0.16	0.17	55.78	55.13
12	1	-1	1	-1	0.65	0.06	0.16	0.09	60.40	60.82
13	1	1	-1	-1	0.65	0.16	0.06	0.09	55.62	55.61

14	-1	-1	-1	1	0.35	0.06	0.06	0.17	68.00	67.67
15	1	-1	1	1	0.65	0.06	0.16	0.17	56.50	55.76
16	0	2	0	0	0.50	0.21	0.11	0.13	50.20	50.58
17	1	1	-1	1	0.65	0.16	0.06	0.17	56.00	55.79
18	0	0	0	-2	0.50	0.11	0.11	0.05	61.13	60.85
19	-1	-1	-1	-1	0.35	0.06	0.06	0.09	56.00	56.44
20	2	0	0	0	0.80	0.11	0.11	0.13	63.00	63.37
21	0	0	0	0	0.50	0.11	0.11	0.13	92.00	92.00
22	-1	1	1	1	0.35	0.16	0.16	0.17	60.90	61.30
23	0	0	0	0	0.50	0.11	0.11	0.13	92.00	92.00
24	1	-1	-1	1	0.65	0.06	0.06	0.17	78.00	78.52
25	0	0	-2	0	0.50	0.11	0.01	0.13	65.00	64.95
26	-1	-1	1	1	0.35	0.06	0.16	0.17	52.59	51.19
27	-1	-1	1	1	0.35	0.06	0.16	0.17	49.99	51.19
28	1	-1	-1	-1	0.65	0.06	0.06	0.09	77.97	77.35
29	-1	1	1	-1	0.35	0.16	0.16	0.09	58.00	57.28
30	-1	1	-1	1	0.35	0.16	0.06	0.17	56.30	55.68

Each factor in this design is studied at five different levels -2, -1, 0, +1, +2 (Table 3) and a set of 30 experiments are carried out. All the variables are taken at a central coded value considered as zero. The experiments with five different concentrations of each significant variable from minimum to maximum ranges are employed simultaneously covering the spectrum of variables for Keratinase production in the central composite design. The full experimental plan with respect to their values in coded form is shown in Table 4. Batch experiments are conducted as per the central composite design for Keratinase production in 250 ml Erlenmeyer flasks. All the experiments are carried out in triplicates and the average value is taken as the response. The CCD experiment is designed using the Design Expert Software package (Version 8.0.7.1, Stat-Ease, Inc., and Minneapolis, USA).

Statistical Analysis and Modelling

This method is suitable for fitting a quadratic surface and it helps to optimize the effective parameters with minimum number of experiments as well as to analyze the interaction between the parameters. In order to determine the existence of a relationship between the factors and response variables, the collected data were analyzed in a statistical manner, using regression. A multiple regression analysis of the data was carried out for obtaining an empirical model that relates the response measured to the independent variables. The quadratic regression models are one of the most widely used in practice. They allow description of the object in a comparatively wide area of the input variables change. A second order polynomial equation is,

$$Y = \beta_0 + \sum_{i=1}^{k} \beta_i X_i + \sum_{i=1}^{k} \beta_{ii} X_i^2 + \sum_{i=1, i<j}^{k-1} \sum_{j=2}^{k} \beta_{ij} X_i X_j \quad (1)$$

Where Y is the measured response, β_0 is the intercept term, β_i are linear coefficients, β_{ii} are quadratic coefficient, $\beta_{ij}$are interaction coefficient and X_i and X_j are coded independent variables.

The following equation was used for coding the actual experimental values of the factors in the range of (−1 to +1):

$$x_i = \frac{X_i - X_0}{\Delta X_i} \quad (2)$$

Where x_i is the coded value of the i^{th} independent variable, X_i the natural value of the i^{th} independent variable, X_0 the natural value of the i^{th} independent variable at the center point, and ΔX_i is the value of step change. Statistical analysis of the data was performed by design package Design-Expert software (Version 8.0.7.1, Stat-Ease, Inc., Minneapolis, USA) to evaluate the analysis of variance (ANOVA), to determine the significance of each term in the equations fitted and to estimate the goodness of fit in each case.

3. Results and Discussion

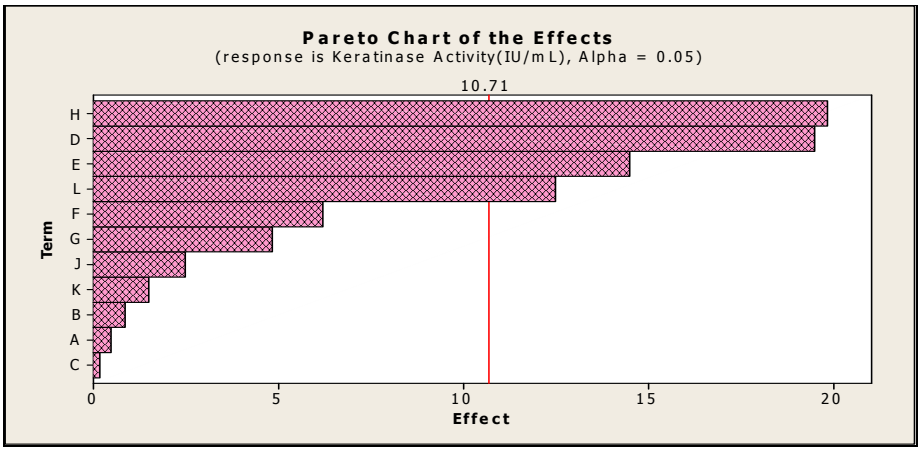

Fig. 1 Pareto chart showing the effect of media components on Keratinase activity

The experimental designs and the results obtained for the twelve experiments with the eleven components are given in Table.2. The twelve run of Plackett-Burman design of experiments show a wide variation in Keratinase activity 18 IU/ml to 64 IU/ml which reflected the importance of optimization to obtain the higher yield. The results are shown in Pareto chart of Fig.1.From the Pareto chart, the variables namely Peptone, KCl, NaCl and K_2HPO_4 are found to be most significant components affecting the Keratinase production and are considered for further optimization using CCD. All the insignificant variables from the PB design are neglected and the optimal concentrations of the four most significant variables are used for further optimization using CCD of RSM to obtain maximum Keratinaseproduction.

For this study, 2_4 full factorial central composite design with sixteen star points, eight axial points and six replicates at the centre points to estimate the experimental error and to have a satisfactory orthogonality for coefficient estimation (all factors at level 0) were employed to fit the second order polynomial model which indicated that 30 experiments were required for this procedure. The coded and actual values of the significant variables are shown in Table.3.The predicted and observed responses along with design matrix are present in Table.4 and the results were analysed by ANOVA.The following second order Polynomial equation describing the correlation between Keratinase and the four variables were obtained.

$$Y=92.000+3.6867A-2.9092B-2.7275C+1.2973D-2.6850AB-1.5700AC-2.5160AD$$

$$+5.5250BC-0.2460BD-1.5540CD-9.0000A^2-8.9013B^2-8.1263C^2-7.14D^2 \quad (3)$$

Table: 5 Analysis of variance (ANOVA) for response surface quadratic model

Source	Coeff Estimate	Sum of Squares	df	Mean Square	F Value	P value Prob> F
Model	92.0000	6715.038	14	479.646	901.663	< 0.0001
A	3.6867	308.738	1	308.738	580.382	< 0.0001
B	-2.9092	192.244	1	192.244	361.390	< 0.0001
C	-2.7275	168.987	1	168.987	317.670	< 0.0001
D	1.2973	37.145	1	37.145	69.827	< 0.0001
AB	-2.6850	106.329	1	106.329	199.883	< 0.0001
AC	-1.5700	36.353	1	36.353	68.339	< 0.0001
AD	-2.5160	89.541	1	89.541	168.324	< 0.0001
BC	5.5250	450.220	1	450.220	846.347	< 0.0001
BD	-0.2460	0.856	1	0.856	1.609	0.2240
CD	-1.5540	34.162	1	34.162	64.220	< 0.0001
A*A	-9.0000	2212.777	1	2212.777	4159.69	< 0.0001
B*B	-8.9013	2164.485	1	2164.485	4068.91	< 0.0001

C*C	-8.1263	1803.985	1	1803.985	3391.22	< 0.0001
D*D	-7.14	1392.673	1	1392.673	2618.02	< 0.0001
Residual		7.979	15	0.532		
Lack of Fit		4.599	9	0.511	0.907	0.5704
Pure Error		3.380	6	0.563		
Corr Total		6723.018	29			

Std. Dev. –0.7294, R^2-0.9988, Mean-65.63, Adj R^2-0.9977, C.V.%-1.113, Pred R^2-0.9944,Adeq Precision-90.293, PRESS - 37.2935

Where Y is the Keratinase activity (IU/ml) respectively A, B, C and D are peptone, NaCl,KCl and K_2HPO_4 respectively. ANOVA for the response surface is shown in Table.5. The independent variables are fitted to the second order model equation and examined for the goodness of fit. The statistical significance of equation is checked by F-test.The results demonstrated that the model is highly significant and is evident from Fischer's F-test with a low probability value (P model>F less than 0.05).The model F-value of 901.663 for Keratinase implies the model is significant. Such a large F-value could occur due to noise and the chance of getting such a high F Value is 0.01% only. Values of Prob>F greater than 0.1 indicates the model terms are not significant. In the present work, the linear effects, the interactive effects and squared effects are significant model terms for Keratinase production. The fit of the model is checked by the coefficient of determination R^2.The coefficient of determination R^2 for Keratinase is calculated as R^2=0.9988 which is nearly equal to 1,indicating that 99.88 % variability of the response could be explained by the model and only about 0.12% of the total variation cannot be explained by the model. This shows that the model is very much suitable for Keratinase production using *Bacillus subtilis NCIM- 2724*by SmF.The predicted R^2 value of Keratinase activity is found to be 0.9944 and is in reasonable agreement with the adjusted R^2 value of 0.9977%.Normally a regression model having an R^2 value higher than 0.90 is considered to have a very high correlation[25].The interactive effects of these variables on Keratinase production is studied by plotting the 3D response surfaces with the vertical axis representing enzyme activity (response) and two horizontal axis representing the coded values of two independent variables, while keeping other variables at their constant level. The three dimensional response surface curves of the calculated response (Keratinase production) with the interactions between the variables are shown in Fig.2to 7**.**

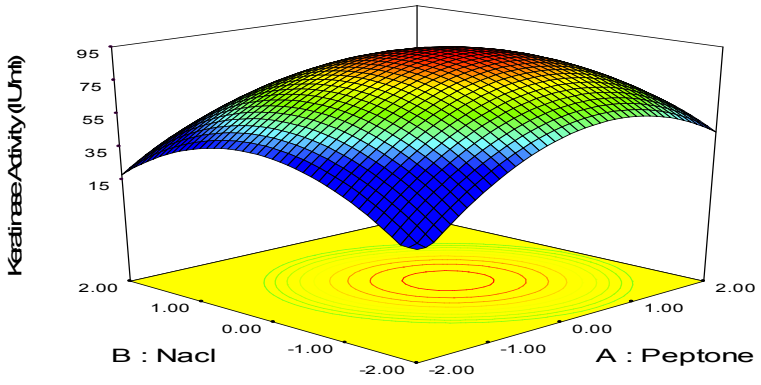

Fig. 2 3D Response surface plot showing interactive effect of Peptone and NaCl on Keratinase activity

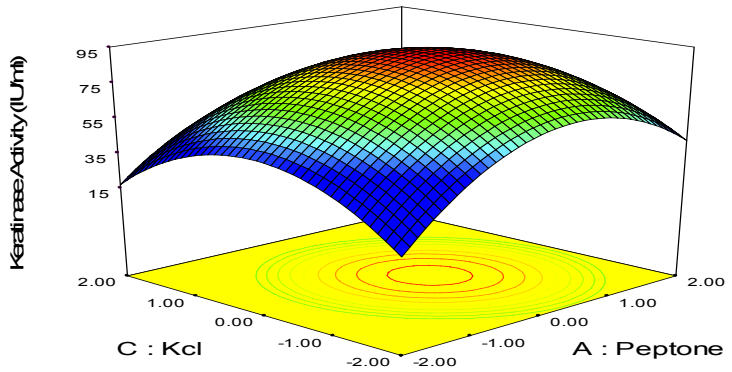

Fig.3 3D Response surface plot showing interactive effect of Peptone and KCl on Keratinase activity

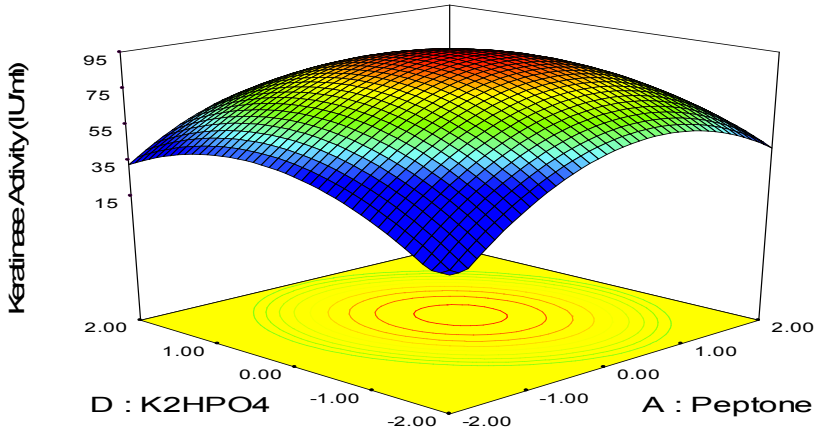

Fig. 4 3D Response surface plot showing interactive effect of Peptone and K_2HPO_4 on Keratinase activity

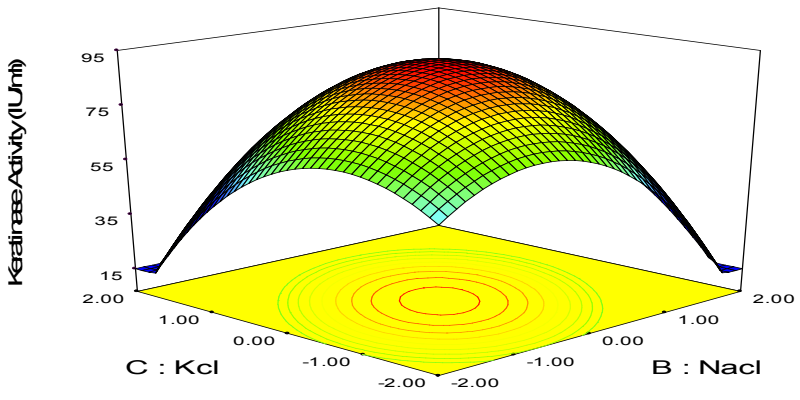

Fig.5 3D Response surface plot showing interactive effect of NaCl and KCl on Keratinase activity

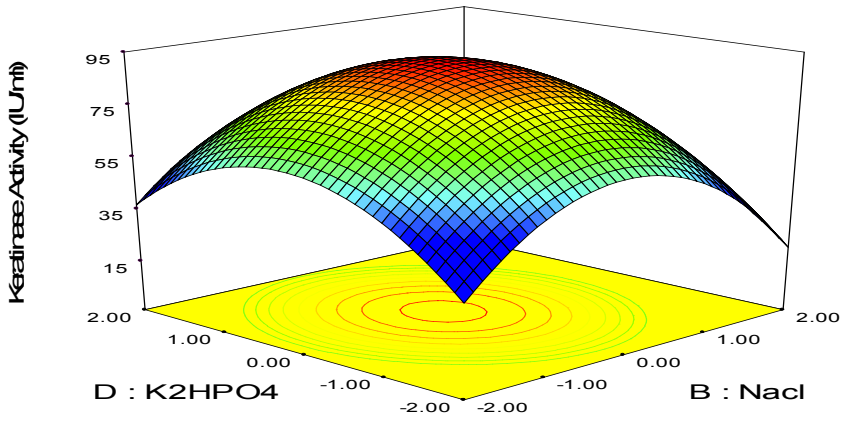

Fig.6 3D Response surface plot showing interactive effect of NaCl and K_2HPO_4 on Keratinase activity

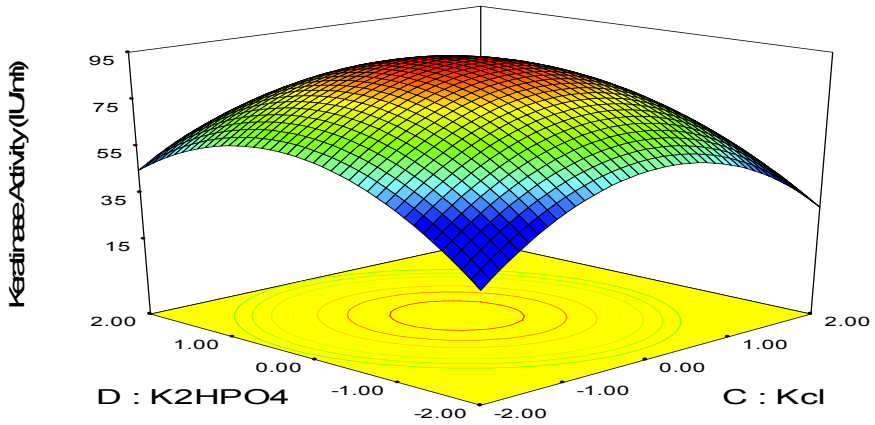

Fig.7 3D Response surface plots showing interactive effect of KCl and K_2HPO_4 on Keratinase activity

Fig. 2 to 4shows the effect of peptone on keratinase production. The keratinase activity increases with increase in the peptone concentration, up to a concentration of 0.53939% w/v peptone and thereafter the enzyme activity decreases with increase in the concentration of peptone. [26] found that the selected nitrogen source depicted maximum and minimum enzyme production at different levels. Such productivity variation may be attributed to nitrogen source mediated regulation of microbial growth and metabolism as it was reported that nitrogen is an essential requirement for growth and production of an enzyme and is an important cellular component mostly as part of the protein.

The same trend is obtained for the study of the effect of NaCl on keratinase production in SmF. The results are shown in Fig. 2, Fig. 5 and Fig. 6. As NaCl concentration is increased from 0.06 % w/v to 0.11 % w/v, the keratinase activity is found to increase and the maximum activity of the enzyme is found at a concentration of 0.09485% w/v of NaCl. For further increase in the concentration of NaCl, thekeratinase activity is found to decrease. Hence 0.09485% w/v of the NaCl concentration is found to be the optimum concentration and is used for further studies.

Fig. 3, Fig. 5, Fig. 7shows the effect of KCl on keratinase activity. As KCl concentration is increased from 0.06 % w/v to 0.11 % w/v, the keratinase activity is found to increase and the maximum activity of the enzyme is found at a concentration of 0.09485% w/v of KCl.

The effect of K_2HPO_4 on keratinase production is shown in the Fig. 4, Fig. 6, Fig. 7. As K_2HPO_4 concentration is increased from 0.13 % w/v to 0.171 % w/v, the keratinase activity is found to increase and a maximum activity of the enzyme is found at a concentration of 0.13384% w/v of K_2HPO_4. For further increase in the concentration of K_2HPO_4 the keratinase activity is found to decrease. Na^+ and K^+ ions are known to be metal activators which protect the enzymes against thermal denaturation and play a vital role in maintaining the active configuration of the enzyme at high temperatures[27].

The maximum predicted yield is indicated by the confined surface in the response surface plot and the optimum values were obtained by solving the second order polynomial equation. The optimum concentrations of the four independent variables in the coded units are found to be A (0.2626), B (-0.3030), C (0.3030) &D(0.0960) and the corresponding uncoded values for maximum keratinase production are:A- Peptone (0.539395% w/v), B - NaCl (0.09485% w/v), C - Kcl (0.09485% w/v) and D - K_2HPO_4 (0.13384% w/v).The predicted values from the regression equation closely agreed with that obtained from experimental values. Fig 8shows that the experimental keratinaseactivity values agree well with the predicted response values.

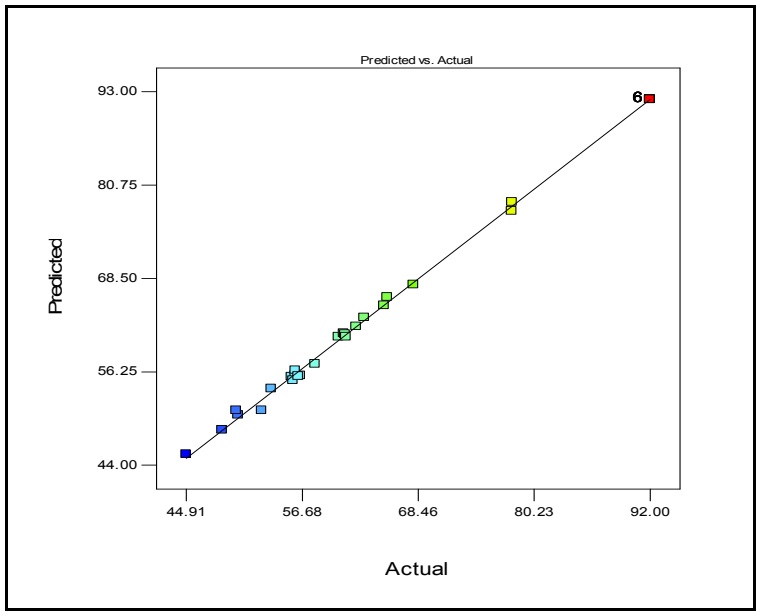

Fig .8 Predicted Vs. Actual (Experimental) Value

3.1 Experimental Validation of the Model

Experimental Validation of the model is tested by carrying out the batch experiment under optimal operation conditions. Experiments are repeated thrice, and the results are compared. The keratinase activity 92.28 IU/ml obtained from experiments was very close to the actual response92.03IU/ml predicted by the regression model, which proved the validity of the model.

4. Conclusion

The results showed the potential use of submerged fermentation for the production of Keratinase using *Bacillus subtilis NCIM 2724*. In this study, the applicability of statistical optimization of placket–Burman design and central composite design of response surface methodology proved to be efficient in determining the significant variables and optimum condition for the production of Keratinase enzyme in submerged fermentation. Among eleven nutrients investigated in the placket–Burman design peptone, NaCl, KCl and K_2HPO_4 were found to be most significant variables. Thereafter a central composite design was employed to find the optimal concentrations of these four nutrients in the fermentation medium in order to maximize Keratinase production. In conclusion, a higher Keratinase activity of 92.28 IU/ml was obtained with the optimized medium comprising of peptone (0.53939%w/v), NaCl (0.09485%w/v),KCl (0.09485%w/v) and K_2HPO_4(0.13384%w/v).

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