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Optimization of Keratinase Production by *Amycolatopsis* sp. Strain MBRL 40 from a Limestone Habitat

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Abstract

Background: The class Actinobacteria accounts for a high proportion of soil microbial biomass and more than half of the bioactive compounds including antibiotics, immunosuppressive agents, antitumor agents and industrial enzymes such as keratinases. Keratinases are modern proteases that can valorise poultry and leather industry wastes and may find applications in prion degradation and treatment of neurodegenerative diseases. A wide diversity of bacteria, actinomycetes and fungi are known to be keratin degraders. Manipur, being a part of Indo-Burma Hotspot, might harbor a rich diversity of bioactive actinomycetes. The objectives of the study are:

a) Isolation of new keratinolytic actinomycetes.

b) Medium optimization by statistical approaches for keratinase production.

Methods: A protease producing actinomycete strain from Hundung limestone quarry at Ukhrul, Manipur, India, was investigated for its keratinolytic activities. Optimization of keratinase production was done using statistical methods namely, Plackett-Burman Design (PBD) and Response Surface Methodology (RSM). Eleven (11) variables were screened using PBD.

Results: The strain showed keratinolytic activity with keratin azure and chicken feather as substrates. Maximum keratinolytic activity was observed at 40°C, pH 7 and 48 h of incubation. Yeast extract, $MgSO_4$, and corn flour were found to affect the response signal positively whereas glucose, $CaCO_3$, K_2HPO_4, NaCl and soyabean meal had negative effects. Yeast extract, cornflour, and soyabean meal were further studied using RSM. A 2.3 fold increase in keratinase production was achieved in submerged fermentation after the use of statistical optimization methods.

Conclusion: On the basis of biochemical properties and 16S rRNA gene sequence analysis, the strain was identified as *Amycolatopsis* sp. strain MBRL 40.

Novelty of the work: There are meagre reports of keratinolytic bacteria from limestone biotopes. In addition, keratinolytic *Amycolatopsis* species are scanty in the literature. This possibly is the first report of a keratinolytic *Amycolatopsis* strain from a limestone habitat.

Keywords: Keratinase; Response surface methodology; Plackett-Burman design; *Amycolatopsis* sp. strain MBRL 40; Manipur; Optimization; Limestone habitat

Introduction

Keratinases (EC 3.4.99.11) are special proteases which attack the highly recalcitrant keratinaceous substrates. They are usually serine or metalloproteases capable of degrading keratin, a fibrous and insoluble structural protein extensively cross-linked with disulphide, hydrogen and hydrophobic bonds [1]. Structurally, keratins are classified as α-keratins (hair, hooves, nails, etc.) and β-keratins (feather, silk fibrion, β-amyloid) [2,3]. β keratins are more readily hydrolyzed than α-keratins [1].

Feathers contain about 80 to 90% keratin on the dry mass basis and are a major waste by-product of the poultry industry [4]. Worldwide, 24 billion chickens are killed annually and around 8.5 billion tonnes of poultry feather are produced [5]. Large amounts of keratin-containing wastes are discharged every year by the poultry, leather and meat processing industries [6]. Feathers represent 5 to 7% of the total weight of mature chickens thereby producing substantial amounts of poultry wastes which are degraded very slowly in nature and can, therefore, become an environmental concern [7]. Keratinous wastes have great potential as a source of protein and amino acids for use as food and feed. However, the current physicochemical processes to produce feather meal are unfriendly to the environment and also destroy certain essential amino acids, yielding a product with poor digestibility and variable nutrient quality [8,9]. However, microbial keratinases can degrade keratin and convert it into environmentally safe and economically nutritious poultry meal, nitrogenous fertilizers [10], biodegradable films and biofuels [11]. Keratinase production by bacteria, actinomycetes, and fungi belonging to the genera *Bacillus*, *Microbacterium*, *Streptomyces*, *Aspergillus*, *Fusarium* and *Amycolaptosis* have been reported earlier [10,12].

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The present investigation aims at the degradation of feather keratin by extracellular keratinase produced by *Amycolatopsis* sp. strain MBRL 40, a protease producing actinomycete isolate obtained from Hundung limestone deposit sites, Ukhrul, India. It also encompasses the optimization of keratinase production using statistical approaches (PBD and RSM).

Materials and Methods

Culture characteristics and growth conditions

A protease producing *Amycolatopsis* sp. strain MBRL 40 was allowed to grow on Starch Casein Nitrate broth (SCNB) and kept incubated in an orbital shaker (Scigenics, India) for 7 days (pH 7, 30°C, 150 rpm).

Keratinase production assays

Keratinase production was carried out using Feather Basal medium-1(FBM-1) containing the following: 0.05% $MgSO_4$, 0.1% K_2HPO_4, 0.3% $CaCO_3$ and 1% chicken feather (pH 7). Cultivation was performed in 250 ml Erlenmeyer flasks containing 50 ml FBM-1 medium and incubated in an orbital shaker at 150 rpm at 30°C for 7 d. The culture broth obtained was centrifuged at 10,000 rpm for 10 min. The supernatant was then used for enzyme assays using keratin azure and chicken feather as substrates.

Keratin azure as substrate

Keratinolytic activity was determined using the modified protocol of Letourneau et al. [13]. The reaction mixture contained 1 ml of supernatant and 10 mg of keratin azure (Sigma) in 1.5 ml of 50 mM phosphate buffer (pH 7). The mixture was incubated at 40°C for 8 h. Absorbance was measured at 595 nm using UV-VIS spectrophotometer (Beckman Coulter, Model no. DU^{R}730). One unit of keratinase activity (KU) was defined as the amount of protease which resulted in an increase of 0.1 absorbance units per hour when measured at 595 nm.

Chicken feather as substrate

Keratinase activity was determined as described by Ramnani et al. [14] with chicken feather as substrate. The assay mixture consisted of 1 ml of the supernatant, 3 ml of 50 mM Phosphate buffer (pH 7) and 20 mg feather. The mixture was incubated at 40°C for 8 h. The reaction was terminated by adding 1 ml of 5% Trichloroacetic acid (TCA). Feather and insoluble residues were removed by filtration through glass wool. Absorbance was measured at 280 nm. An increase of 0.01 absorbance unit denotes 1 unit of enzyme activity (KU/ml).

Medium optimization for keratinase production

Keratinase production by *Amycolatopsis* sp. strain MBRL 40 was initially carried out in the FBM-1 medium. The medium optimization was carried out in the following three steps.

Optimization by one variable at a time approach

The effects of pH in the range of 5 to 13 on keratinase production were studied by adding appropriate buffers (50 mM) in FBM-1 medium *viz*. Citric acid/ Na_2HPO_4 (pH 5, 6 and 7), Tris-HCl (pH 8 and 9), Glycine-NaOH (pH 10 and 11) and KCl/NaOH (pH 12 and 13). Effects of inoculum size (1 to 10% v/v) and inoculum age (3 to 7 d) on enzyme production were also monitored. Enzyme production was carried out at different temperatures ranging from 30°C to 70°C (temperature intervals of 10°C).

Medium optimization was also studied by varying the components of inorganic salts viz. $MgSO_4.7H_2O$ (0.05%, 0.07%, 0.08%, 0.1%, w/v), $CaCO_3$ (0.03%, 0.05%, 0.08%, 0.1%, 0.15%, 0.2%, 0.25%, 0.3%, w/v) and K_2HPO_4 (0.1%, 0.15%, 0.2%, 0.25%, 0.3%, w/v) in the FBM-1 medium.

The effects of various carbon sources *viz*. glucose, sucrose, lactose, starch, maltose, fructose, mannitol, mannose, corn flour, rice bran, galactose (0.5%, w/v each) and nitrogen sources viz. yeast extract, peptone, NH_4Cl, urea, tryptone, beef extract, $NaNO_2$, soyabean meal (0.5% each w/v) on keratinase production were studied (Figures 1 and 2).

Optimization of keratinase production using statistical approaches

After the initial selection of various physical and nutritional factors *viz*. pH, temperature, inoculum size, inoculums age, C sources, N sources, and inorganic salts by one variable at a time approach (OVAT), the optimised medium was designated as FBM-2. The medium was further optimised using statistical approaches *viz*. Plackett-Burman design (PBD) and Response Surface Methodology (RSM) and the modified media were designated as FBM-3 (after PBD) and FBM-4 (after RSM) respectively.

Screening of signal parameters using PBD

The effects of 11 factors viz. glucose, yeast extract, $CaCO_3$, $MgSO_4$, K_2HPO_4, feather, NaCl, corn flour, soyabean meal, inoculum size, and inoculum age were studied using the FBM-2 medium. All the variables were investigated at two widely spaced levels (high and low levels) (Table 1). The minimum and maximum ranges selected for keratinase

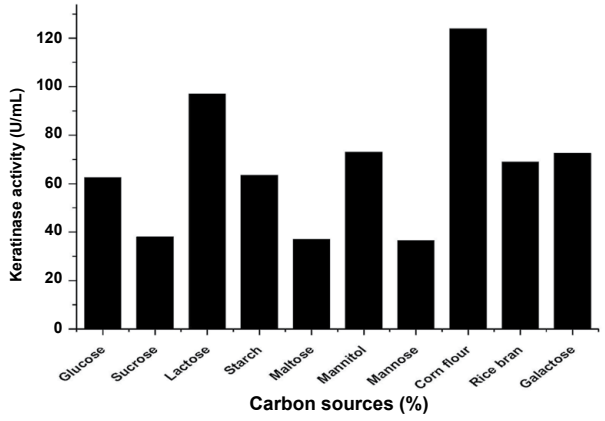

Figure 1: Effects of different carbon sources on keratinase production.

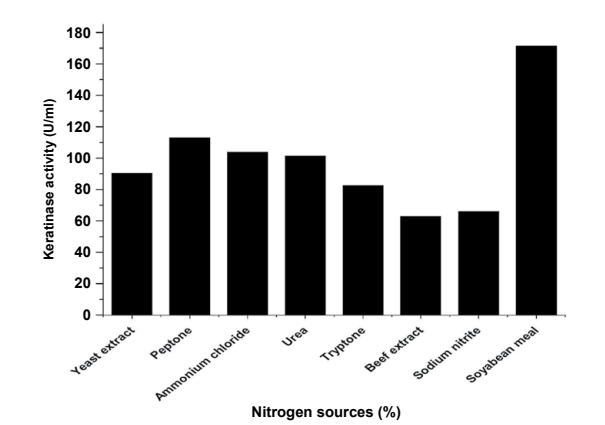

Figure 2: Effects of different nitrogen sources on keratinase production.

production were studied. Design expert® software 6.0 (Stat-Ease, Inc., Minneapolis, USA) was used to generate a set of 12 experimental designs.

Maximum and minimum production was studied in SCNB kept incubated in an orbital shaker for 60 h at 30°C and 150 rpm. Unpaired student t-test and p-value of each factor were calculated for determining the effect of media components on the enzyme production (Table 2).

Response Surface Methodology (RSM)

The optimum concentrations and interactions of signal parameters *viz*. yeast extract, cornflour and soyabean meal were studied by RSM. FBM-3 medium, designed by PB method, was further modified by RSM using Central Composite Design (CCD). The medium was studied at five different levels (-α, -1, 0, +1 and +α). A set of 20 experiments were generated by Design Expert® software 6.0 (Stat-Ease). The minimum and maximum ranges of the variables were investigated and the full experimental design with respect to their actual and coded forms is listed in Table 3.

The average maximum keratinase activity was taken as the dependent variable. A multiple regression analysis was performed on the data obtained, and a quadratic model was expressed as the equation:

$$Y=\beta_0+\Sigma\beta_i X_i+\Sigma\beta_i X_i^2+\Sigma\beta_{ij} X_i X_j$$

Where, Y is the predicted response, β_0 is the intercept, β_i is the linear coefficient, β_{ij} is the interaction coefficient. The equation was analysed by Design Expert and responses of variables were analysed by three-dimensional and contour plots.

Variable (%) w/v	High level (+)	Low level (-)
Glucose	1.0	0.1
Yeast extract	0.1	0.01
Feather	2.0	0.5
$CaCO_3$	0.3	0.0
$MgSO_4$	0.1	0.0
K_2HPO_4	3.0	0.0
Inoculum size	5.0	1.0
Inoculum age (days)	7.0	3.0
NaCl	2.0	0.5
Corn flour	1.0	0.0
Soyabean meal	1.0	0.5

Eleven (11) variables shown above were chosen for analysis in PBD

Table 1: Maximum and minimum parameters selected for Plackett-Burman (PB) run.

Validation of the experimental model

In order to validate the above model, 6 random experiments were set up according to the conditions predicted by the model. Experiments were conducted with varying concentrations of yeast extract (A), corn flour (B) and soyabean meal (C) (Table 4). After validation, the optimised medium was designated as FBM-4.

Time course of keratinase production

Enzyme production was carried out in 50 ml of FBM-4 medium for 72 h (pH 7, 30°C, 150 rpm). Samples were withdrawn at intervals of 12 h and enzyme production was assayed.

Results

Keratinase production

A protease producing *Amycolatopsis* sp. strain MBRL 40 could produce keratinase, and the enzyme production was observed after 60 h of incubation in the FBM-1 medium.

Enzyme assays

The initial keratinolytic activity of the strain in FBM-1 medium was 5.19 KU/ml and 165.66 KU/ml respectively when keratin azure and chicken feather were used as substrates.

Optimization by one variable at a time approach

Maximum keratinase production was observed at pH 7 and 40°C, inoculum size of 5% (v/v) and inoculum age of 7 d. Among the inorganic salts, K_2HPO_4 had the most positive effect on keratinase

Factors	Mean total keratinase production (U/ml) at +1 level	Mean total keratinase production (U/ml) at -1 level	Student's t-test	p-value	Remarks
Glucose	1289.6 ± 22.5	1361.3 ± 47.9	-2.34	0.079	Negative
Yeast extract	1589.3 ± 19.7	1061.8 ± 51.7	16.5	<0.0001	Positive
Feather	1181.8 ± 39.6	1469.3 ±31.7	-9.82	0.0003	Negative
$CaCO_3$	1284.2 ± 23.5	1403 ± 60.7	-4.12	0.0073	Negative
$MgSO_4$	1600.3 ± 65.4	1050.8 ± 7.9	14.4	<0.0001	Positive
K_2HPO_4	1252.5 ± 51.7	1354.9 ±119.6	-1.36	0.1227	Negative
Inoculum size	1547.3 ± 55.9	1103.8 ± 38.3	11.3	<0.0001	Positive
Inoculum age	1477.2 ± 12.3	1174.0 ± 55.6	9.222	0.0003	Positive
NaCl	1157.3 ± 51.9	1493.8 ±8.4	-11.1	<0.0001	Negative
Corn flour	1692.2 ± 43.6	959 ± 22.3	25.9	<0.0001	Positive
Soyabean meal	1128.0 ±41.0	1523.2 ± 46.5	-11.0	<0.0001	Negative

Yeast extract, $MgSO_4$, Inoculum size, Inoculum age and Corn flour enhanced keratinase production whereas Glucose, Feather, $CaCO_3$, K_2HPO_4, NaCl and Soyabean meal decreased keratinase production

Table 2: Screening of signal parameters on the basis of student's t-test and p values using PBD.

Variables	Actual	Coded	Actual	Coded (in percent)	Actual (in percent)	Coded	Actual	Coded	Actual	Coded
Yeast extract	0.0	-α	0.05	-1	0.13	0	0.20	+1	0.25	+α
Corn flour	0.43	-α	0.70	-1	1.10	0	1.50	+1	1.77	+α
Soyabean Meal	0.00	-α	0.10	-1	0.30	0	0.50	+1	0.64	+α

Table 3: Experimental ranges and levels of the three independent variables used in RSM in terms of actual and coded factors.

production. Corn flour was found to be the best supplementary C source, and fructose was the least satisfactory C source. Soyabean meal was the best and beef extract the worst supplementary N source. After OVAT, the medium obtained was designated as FBM-2 which contains 1% (w/v) chicken feather, 0.3% (w/v) $CaCO_3$, 0.05% (w/v) $MgSO_4$, 0.1% (w/v) K_2HPO_4, 1% (w/v) corn flour, 0.5% (w/v) soyabean meal.

Plackett-Burman design

Among the 11 variables tested, corn flour showed the highest positive signal followed by $MgSO_4$, yeast extract, inoculum size, and inoculum age whereas $CaCO_3$, K_2HPO_4, feather, NaCl and soyabean meal showed negative effects (Table 2).

High level of feather (2%) influenced the enzyme production negatively. After analysis of PBD results, the optimised medium was designated as FBM-3 consisting of 0.5% (w/v) chicken feather, 0.1% (w/v) yeast extract, 0.1% (w/v) $MgSO_4$, 1% (w/v) corn flour and 0.5% (w/v) soyabean meal. FBM-3 was then further optimised by RSM.

Optimization by response surface methodology (RSM)

The effect of three factors *viz*. yeast extract, cornflour and soyabean meal were optimized by RSM and keratinase production was monitored using Central Composite Design (CCD). Experimental and predicted values for keratinase production are presented in Table 5a.

On regression analysis of experimental data, the following quadratic equation was obtained for keratinase production.

$$Y=196.92+34.68*A-35.51*B+88.75*C+2.75*A^2-21.37*B^2+16.47*C^2+24.06*A*B+12.81*A*C-10.94*B*C$$

Where A, B and C represent concentrations of yeast extract, corn flour and soyabean meal respectively. All three linear coefficients, A, B, C and squared term B of the model were significant for keratinase production. The R^2 value of 0.9371 for keratinase production indicated the accuracy of the model. The ANOVA values for keratinase production indicated that the model was significant (Table 5b).

One factor, contour, and 3D response surface curves were plotted to study the interactions among the three factors in order to determine the optimum concentration of each for maximum keratinase production. An increase in corn flour (CF) concentration decreased keratinase production whereas an increase in yeast extract (YE), and soyabean meal (SM) concentration increased keratinase production (Figures 3A-3C). Between the two widely spaced concentration levels, the concentration of CF at the midpoint results in maximum keratinase production. From individual 3D interaction curves, it could be inferred that combinations of high YE and medium CF; high YE and high SM; and high SM and medium CF favoured optimal enzyme production. However, when overall interactions are considered, the maximal enzyme production occurred at medium levels of YE and CF, and high level of SM i.e., 0.13% (w/v) yeast extract, 1.1% (w/v) corn flour and 0.5% (w/v) soyabean meal.

Validation of the experimental model

The model was validated for all three variables within the design space using a set of 6 random experiments and observed keratinase production values were found to be close to the predicted values. Therefore after RSM, the optimised medium was designated as FBM-4 consisting of 0.5% (w/v) chicken feather, 0.13% (w/v) yeast extract, 1.1% (w/v) corn flour and 0.5% (w/v) soyabean meal (Table 6).

Run	A(Yeast extract)	B(Corn flour)	C (Soyabean meal)
1.	0.2	0.91	0.5
2.	0.5	1.1	0.3
3.	0.13	1.5	0.3
4.	0.13	1.1	0.3
5.	0.13	1.1	0.1
6.	0.13	1.1	0.5
7.	0.2	1.1	0.3

Table 4: Experiments conducted for validation of the response surface model.

Run No.	Yeast extract	Corn flour	Soyabean meal	Keratinase production (U/ml)	
(a) Experimental design for RSM studies				Observed	Predicted
1.	-1	-1	-1	130.00	132.79
2.	+1	-1	-1	130.00	128.40
3.	-1	+1	-1	50.00	35.51
4.	+1	+1	-1	105.00	127.37
5.	-1	-1	+1	325.00	306.55
6.	+1	-1	+1	335.00	353.40
7.	-1	+1	+1	160.00	165.52
8.	+1	+1	+1	307.50	308.62
9.	-α	0	0	130.00	146.76
10.	+α	0	0	285.00	263.01
11.	0	-α	0	195.00	196.21
12.	0	+α	0	83.50	76.75
13.	0	0	-α	105.00	100.85
14.	0	0	+α	395.00	392.77
15.	0	0	0	165.00	196.92
16.	0	0	0	210.00	196.92
17.	0	0	0	260.00	196.92
18.	0	0	0	150.00	196.92
19.	0	0	0	220.00	196.92
20.	0	0	0	175.00	196.92

(b) ANOVA values for keratinase production	
Term	**Keratinase**
F-value	16.54
P>F	0.0001
Mean	195.80
R^2	0.9371
Adjusted R^2	0.8804
Coefficient of variance	16.67
Adequate precision	15.483

Table 5: Experimental design of RSM studies using three independent variables with six centre points, showing observed and predicted values of keratinase production (a) and ANOVA analysis (b).

Yeast extract (%)	Corn flour (%)	Soyabean meal (%)	Keratinase activity(U/ml)	
A	B	C	Predicted	Observed
0.2	0.91	0.5	308.19	303.25
0.05	1.1	0.3	164.9	187.4
0.13	0.7	0.3	211	198.6
0.13	1.5	0.3	140	158.3
0.13	1.1	0.1	124.5	107.6
0.13	1.1	0.5	302	318.2

Table 6: Validation of the response surface model within the design space.

Time course of keratinase production

Time course of enzyme production was studied up to 72 h in the optimized FBM-4 medium and maximum enzyme production of 395.00 U/ml was achieved after 48-60 h of incubation (Figure 4).

Discussion

Optimization of keratinase production medium components by using statistical methods such as RSM has gained increasing popularity due to significant improvements in enzyme production titre, cost effectiveness, easy applicability, reliability and validity [15]. Actinomycete isolates have been reported to produce a large number of extracellular enzymes, of which keratinases are of particular significance in feather degradation and leather industry [16].

MBRL 40 produced maximum keratinase at pH 7 and 40°C. Similarly, *Streptomyces albus* has been reported to produce maximum keratinase production at pH 7 and 40°C [17]. Strain MBRL 40 exhibited maximum keratinase production at an inoculum size of 5% (v/v) whereas *Bacillus weihenstephanensis* showed optimal keratinase production at an inoculum size of 2% (v/v) [18]. Strain MBRL 40 exhibited highest and lowest enzyme activities when corn flour and fructose were used as carbon sources respectively. This is in contrast with an earlier report on keratinase production in which fructose enhanced enzyme production in *Bacillus licheniformis* [19]. Cai and Zheng [20] have reported that corn flour has a positive effect on enzyme production in *Bacillus subtilis*. Strain MBRL 40 also exhibited highest and lowest enzyme activities when soyabean meal and beef extract were used as nitrogen sources respectively. This is in contrast with the report of Tiwary and Gupta [19] in which beef extract was found to enhance the enzyme production in *Bacillus licheniformis*. In the PB experiment, corn flour, yeast extract, and $MgSO_4$ were found to exert positive effects while $CaCO_3$, K_2HPO_4, NaCl and soyabean meal had negative effects on enzyme production by MBRL 40. The addition of Mg^{2+} has been reported to increase enzyme production in *Bacillus* sp [21,22] and a decrease in keratinase production in *Serratia marcescens* [23]. K_2HPO_4 has been reported to show a positive signal in *Bacillus* sp. which is in contrast with our findings. Ca^{2+} showed negative effects in *Bacillus* spp. which are similar to our findings [20-22]. Maximum keratinase production by MBRL 40 occurred at 0.5% feather concentration which is in contrast with the finding in *Bacillus* sp. JB 99 in which maximal keratinase production took place at 1.0% feather concentration [21].

After RSM, maximum keratinase production by MBRL 40 was 395 KU/ml in presence of 0.5% (w/v) chicken feather, 0.13% (w/v) yeast extract, 1.1% (w/v) corn flour and 0.5% (w/v) soyabean meal. This titer is much higher in comparison to earlier reports in which *Streptomyces pactum* and *Bacillus* sp. produced 64 and 60 U/ml [20,24]. Thus, after the use of statistical methods, a 2.3 fold increase in keratinase production was achieved by MBRL 40 in comparison to un-optimized conditions. This keratinolytic strain could be useful for biotechnological processes involving keratin hydrolysis.

Conclusion

There are meagre reports of keratinolytic bacteria from limestone biotopes. In addition, keratinolytic *Amycolatopsis* species are scanty in the literature. This possibly is the first report of a keratinolytic *Amycolatopsis* strain from a limestone habitat.

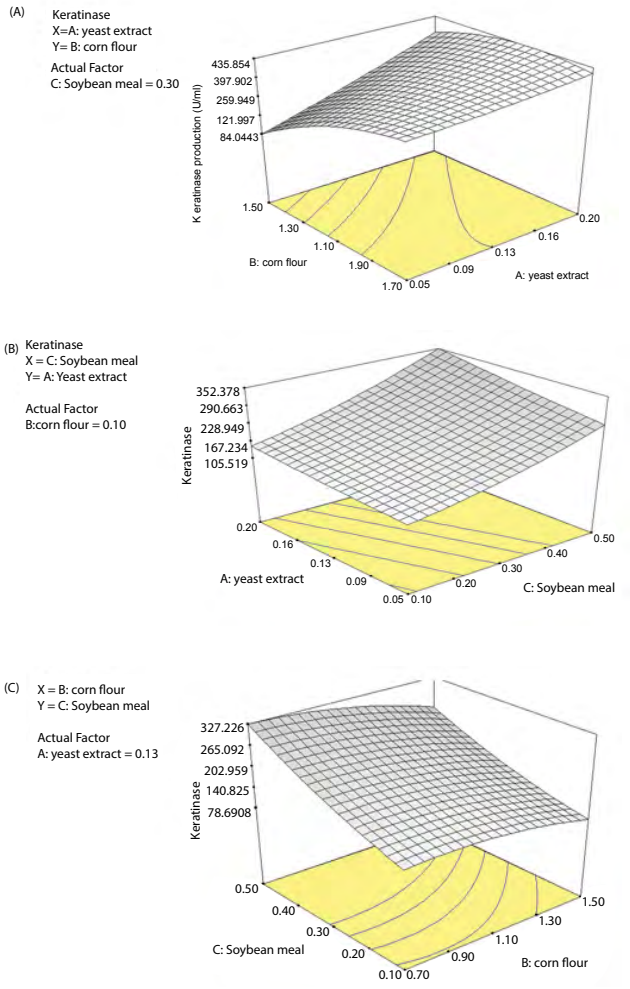

Figure 3: Response Surface Graph for keratinase production: (A) Interaction between corn flour and yeast extract (B) Interaction between soyabean meal and yeast extract (C) Interaction between soyabean meal and corn flour.

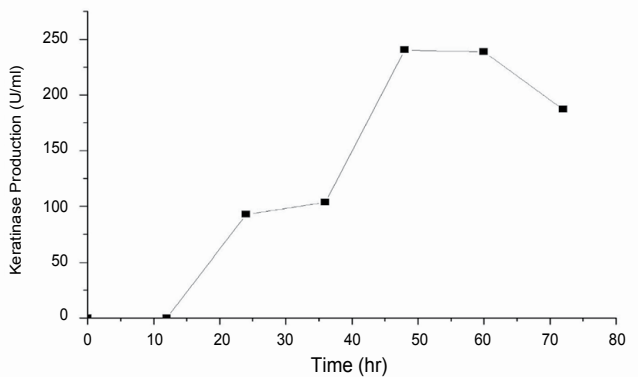

Figure 4: Time course of keratinase production in FBM-4 medium.

Acknowledgements

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Author's Contributions

Debananda S. Ningthoujam initiated the project idea, supervised the study and project management, and wrote the final manuscript. Laishram Jaya Devi, P

Jolly Devi, S Sundari Devi and N Betterson conducted the experiments. Pintubala Kshetri did the statistical analyses. LJD wrote the initial manuscript. K Tamreihao and Saikat Mukherjee contributed through critical reviews and comments and helped in editing the initial versions of the manuscript.

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