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Medium formulation and optimization for improving protease production by *Streptomyces* sp. LCJ17A

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Abstract: **Medium formulation using low cost substrates and suitable culture conditions to optimize protease production were studied by *Streptomyces* sp. The fermentation medium for the production of alkaline protease by *Streptomyces* sp. LCJ17A was optimized with glucose as a carbon source, casein as a nitrogen source and red gram husk as an inducer. The optimized values showed that glucose at 3.5 g/L enhances the yield of protease up to 68.3±1.2 U/mL, casein at 0.5 g/L maximizes the protease production up to 71.72±1.2 U/mL and red gram husk at 2.5 g/L yields 73.02±2.1 U/mL. The protease production was 2.5 fold higher compared to that obtained using the unoptimized medium. Through statistical optimization by using Response Surface Methodology, the enzyme yield was further increased to 88.2±0.9 U/mL. This result indicates that the use of glucose, casein and red gram husk as suitable substrates for maximum protease yield and the enzyme activity from the cheaper source enhances the stability of the protease enzyme for industrial applications.**

Keywords: **Alkaline protease, Starch casein broth, submerged fermentation, Inducer, optimum culture conditions and *Streptomyces* sp. LCJ17A.**

I. INTRODUCTION

The microorganisms involved in the production of industrially important enzymes has stimulated interest for their exploitation in large scale production of extracellular enzymes [1]. In industries, proteases produced from microbes are most commonly used [2]. The proteases are being used for meat tenderization, baking, cheese making, brewing, de-hairing, production of detergents, leather, silk and pharmaceuticals [3], [4], [5]. A variety of microorganisms such as bacteria, yeast, mold and actinomycetes are known to produce protease enzyme [6]. *Streptomyces* sp. are the most important group of Actinobacteria with G+C content of the DNA [7]. To increase the yield and to make the protease enzyme industrially and commercially viable, optimization of production medium is required. For medium optimization, the one at a time approach is frequently used to obtain the maximum level of enzyme in fermentation system. The optimization experiments are usually performed using both non-statistical one-factor-at-a-time [8], [9] in the statistical experimental design approaches [10], [11]. This technique ignores the combined interactions among the variables and also time consuming [12]. Response surface methodology (RSM) are widely used to select the significant variables and obtain the optimal levels, respectively [13], [14]. Optimization of selected, highly influencing factors can be done using response surface methodology with either central composite design (CCD) or Box-Behnken Design experiments [15]. The present study is aimed to optimize a suitable medium and favorable culture conditions for increasing extracellular protease production by *Streptomyces* sp. LCJ17A under submerged fermentation.

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II. MATERIALS AND METHODS

Medium optimization in Submerged Fermentation (Smf):

The composition of liquid medium for production of protease by submerged fermentation. Production of protease was studied in six different basal media namely Modified nutrient glucose broth [16], Starch casein broth [17], Protease production broth [18], Gelatin broth [19], Malt yeast extract broth and Glycerol- peptone salt broth [20]. Among the 6 different media, the starch casein broth (SCB) was selected for the production. This medium increased the growth and production of protease when *Streptomyces* sp. LCJ17A was inoculated.

Starch casein broth contained the following components per liter (g/L): Starch– 10 g, Casein – 3g, KNO_3 – 2g, NaCl – 2g, K_2HPO_4 – 2g, $MgSO_4.7H_2O$ – 0.05g, $CaCO_3$ – 0.02g and $FeSO_4.7H_2O$ – 0.01. About 100 mL of medium was dispensed into 250 mL conical flask and autoclaved at 121˚C for 15mins. Nalidixic acid (25 µg/mL) was added to avoid bacterial contamination. One mycelial disc (4mm) was inoculated into the conical flask, under sterile condition and incubated in the rotary shaker at 120 rpm at room temperature. 10 mL of the culture filtrate was taken at intervals of every 48 hours and centrifuged at 10,000 rpm for 10 mins. The supernatant was used as a crude enzyme solution for protease activity. The protease assay was carried out by using standard protocol [21], [22].

Optimization of protease production under submerged fermentation:

The influence of different factors on the production of protease was carried out in liquid culture medium in separate flasks, examining one factor at a time, keeping all other variables constant. Once the optimization had been done with respect to a factor, it was incorporated into the experiment for the optimization of the next factor. The effect of different carbon sources, nitrogen sources and inducers were studied. The concentration of carbon, nitrogen and inducers in starch casein broth were optimized after selecting the best carbon, nitrogen and inducer. High protease activity meant that the organism was capable of producing high amounts of protease in that particular factor.

Effect of carbon source and its concentration on protease production:

The protease production by the selected isolate *Streptomyces* sp. LCJ17A in starch casein broth was optimized by supplementing different carbon sources like glycerol, glucose, sucrose, fructose, maltose, starch, mannitol and lactose at 1 g/L. Simultaneously, a control medium without the addition of any carbon sources was maintained. After screening for the carbon source favoring maximum protease production, the best carbon source concentration in the range of 0.5 to 5.0 g/L was studied.

Effect of nitrogen source and its concentration on protease production:

The suitable nitrogen source for protease production by *Streptomyces* sp. LCJ17A was studied using organic (peptone, casein, urea and yeast extract) and inorganic nitrogen sources (ammonium nitrate, ammonium sulphate and sodium nitrate) in starch casein broth (SCB) at 5 g/L. These nitrogen sources replaced the original nitrogen source available in the medium. Simultaneously a control medium was also maintained without the addition of any nitrogen sources. After screening for the nitrogen source favoring maximum protease production, the best nitrogen source concentration ranging from 0.5 to 5.0 g/L was evaluated.

Effect of natural inducers and its concentration on protease production:

Starch casein broth was amended with different natural inducers such as groundnut oil cake, sesame oil cake, green gram husk, black gram husk and red gram husk separately at the concentration of 5 g/L. The best inducer which yields higher amount of protease was further optimized at the concentration ranging from 0.5 - 5.0 g/L by *Streptomyces* sp. LCJ17A and the experimental set up was incubated at room temperature under shaking condition (120 rpm) for 12 days.

Experimental design optimization and protease production:

The statistical optimization in medium formulation was to determine the optimal levels of three variables, viz. Glucose (X_1), Casein (X_2) and Red gram husk (X_3) on protease production from *Streptomyces* sp. LCJ17A. Central composite design (CCD) was adopted for improving total protease production. To analyze the experimental design, 'Design-Expert_11.0.6.', StatEase, Inc., (Minneapolis, MN, USA) was used.

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Glucose, casein and red gram husk were studied at three different levels (-1, 0, +1) in the design. A set of 29 experiments was carried out for a 2^3 CCD with three factors, including six center points. All the three variables, glucose, casein and red gram husk were taken at a central coded value considered as zero. The minimum and maximum ranges of variables investigated and the full experimental plan with respect to their values in actual and coded form are listed in TABLE I. After completing the experiments, the average maximum protease yield was taken as the dependent variable or response (*Y*). By the multiple regression procedure, a second-order polynomial equation was fitted to the data. This resulted in an empirical model that related the response measured to the independent variables of the experiment.

TABLE I: EXPERIMENTAL RANGE AND LEVELS OF THE THREE INDEPENDENT VARIABLES USED IN RSM IN TERMS OF ACTUAL AND CODED FACTORS

Range of levels							
Variables (g/L)	**Symbol**	**Actual**	**Coded**	**Actual**	**Coded**	**Actual**	**Coded**
Glucose	X_1	2.5	-1	3.5	0	4.5	+1
Casein	X_2	0.25	-1	0.5	0	0.75	+1
Red gram husk	X_3	1.5	-1	2.5	0	3.5	+1

The model equation for three-factor system is:

$$Y = b_0 + b_1X_1 + b_2X_2 + b_3X_3 + b_{11}X_1^2 + b_{22}X_2^2 + b_{33}X_3^2 + b_{12}X_1X_2 + b_{23}X_2X_3 + b_{13}X_1X_3$$

with Y, predicted response; b_0, intercept; b_1, b_2, b_3 linear coefficients; b_{11}, b_{22}, b_{33} squared coefficients; b_{12}, b_{13}, b_{23} interaction coefficients. Design Expert Software, using the above model to obtain the optimum concentration of the medium components, was then used to generate response surface graphs.

III. RESULTS AND DISCUSSION

Isolation:

Streptomyces sp. LCJ17A was isolated from the sediments of Pichavaram Mangrove. They were characterized morphologically, biochemically and they were screened to confirm for their proteolytic activity using skim milk agar medium and further it was characterized using molecular methods (16S rRNA sequencing) and acquired accession number for the strain *Streptomyces* sp. LCJ17A (accession number: KU870434), was selected for further production process.

Selection of liquid medium for protease production:

Selection of a suitable liquid medium for the production of protease by *Streptomyces* sp. LCJ17A was carried out using six different media i.e., Modified nutrient glucose broth, Starch casein broth, Protease production broth, Gelatin broth, Malt yeast extract broth and Glycerol- peptone salt broth. Protease activity was observed with the respect to the different media. Initiation of actinomycete growth was observed after 2 days of inoculation. Among the six media tested, starch casein broth (SCB) favored maximum protease production and thus, it was selected for further optimization to enhance protease yield.

Submerged Fermentation:

Effect of carbon sources and their concentration on protease activity

Effect of different carbon sources (glucose, sucrose, glycerol, lactose, maltose, mannitol, fructose and starch) and their concentration (0.5- 5.0 g/L) on the production of protease by *Streptomyces* sp. LCJ17A in SCB was studied. The results showed that *Streptomyces* sp. LCJ17A exhibited higher protease production in the liquid medium containing glucose with protease activity: 52.60±1.8 U/mL at 1 g/L than other carbon sources (TABLE II).

TABLE II: EFFECT OF VARIOUS CARBON, NITROGEN AND INDUCER SOURCES ON PROTEASE PRODUCTION BY *STREPTOMYCES* SP. LCJ17A ON 8TH DAY AT 37°C AT 120 RPM

Nutrient source	**Protease yield U/mL**
Carbon source (1g/L)	
Glucose	52.60±1.8
Starch	42.17±1.8
Lactose	34.94±2.1

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Sucrose	33.69±1.5
Glycerol	31.40±1.5
Fructose	30.83±1.8
Mannitol	24.64±1.7
Maltose	21.09±1.2
Nitrogen source (5g/L)	
Casein	53.72±1.1
Yeast Extract	26.12±1.1
Sodium Nitrate	24.99±1.3
Peptone	22.13±1.2
Ammonium Nitrate	22.12±1.0
Urea	21.20±1.1
Ammonium Sulphate	19.03±0.9
Inducers (5g/L)	
Red gram husk	62.14±1.8
Sesame oil cake	24.56±1.0
Groundnut oil cake	23.07±1.2
Green gram husk	21.98±1.5
Black gram husk	20.40±1.7

The results on the effect of different concentrations of glucose on the protease production by *Streptomyces* sp. LCJ17A is depicted in Fig. 1. Among the different concentrations tested, 3.5 g/L of glucose enhanced maximum yield of protease with 68.3±1.2 U/mL of protease activity by *Streptomyces* sp. LCJ17A on the 8th day of inoculation. The production of protease increased with increase in the concentration of best carbon sources but declined when the concentration was increased beyond 4.0 g/L. Physical and nutritional factors influence the protease production and therefore optimization of the factors is essential to improve the protease yield [23]. In previous studies, protease production by using glucose and wheat bran as carbon source enhanced the yield by *Bacillus* sp. [24]. Glucose and peptone served as a carbon and nitrogen source yields maximum of 4.548 g/L, which is lesser than the present study yield [25].

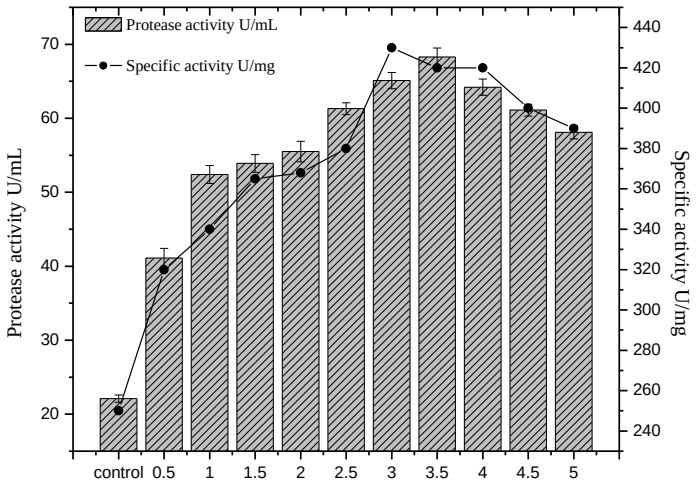

Fig 1: EFFECT OF CONCENTRATION OF GLUCOSE FOR PROTEASE PRODUCTION BY *Streptomyces* sp. LCJ17A.

Effect of nitrogen sources and their concentration on protease activity:

Effect of different nitrogen sources and their concentration on the production of protease by *Streptomyces* sp. LCJ17A was studied using different organic (casein, urea, peptone and yeast extract) and inorganic nitrogen sources (ammonium sulphate, ammonium nitrate and sodium nitrate) in SCB. The results showed that the protease production was maximum with organic nitrogen sources for *Streptomyces* sp. LCJ17A (TABLE II). Addition of casein (5 g/L) to the medium enhanced maximum protease production with 53.72±1.1 U/mL by *Streptomyces* sp. LCJ17A. The peak protease activity was observed on the 8th day of inoculation and declined thereafter for the strain *Streptomyces* sp. LCJ17A.

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The effect of different concentrations of casein (0.5 to 5.0 g/L) by *Streptomyces* sp. LCJ17A was studied in SCB and results showed that a maximum protease activity of 71.72±1.2 U/mL was observed at 0.5 g/L of casein for *Streptomyces* sp. LCJ17A depicted in Fig. 2. The actual nitrogen source, casein along with potassium nitrate in SCB medium enhanced the yield of protease at the concentration of 0.5 g/L comparing to the other nitrogen sources. In another study, the alkaline protease production was influenced to a great extent by the dominance of casein along with other nitrogen sources by *Virgibacillus pantothenticus* [26]. In some optimization study, different organic and inorganic nitrogen sources were used for the production of protease. Inorganic nitrogen sources showed higher protease production compared to organic nitrogen sources [27].

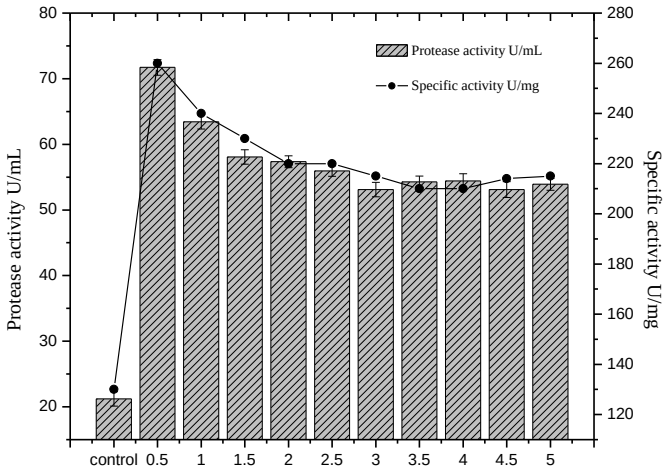

FIG 2: EFFECT OF CONCENTRATION OF CASEIN FOR PROTEASE PRODUCTION BY *Streptomyces* sp. LCJ17A

Effect of natural inducer and their concentration on protease activity:

The effect of different natural inducers such as green gram husk, black gram husk, red gram husk, groundnut oilcake and sesame oilcake on the production of protease were studied. Addition of natural inducer (red gram husk) to the medium favoured maximum protease activity of 62.14±1.8 U/mL at 5 g/L for *Streptomyces* sp. LCJ17A (TABLE II).

The effect of different concentrations of red gram husk (0.5 to 5.0 g/L) by *Streptomyces* sp. LCJ17A was studied and results showed that a maximum protease activity of 73.02±2.1 U/mL was observed at 2.5 g/L of red gram husk by *Streptomyces* sp. LCJ17A showed in Fig. 3. Addition of red gram husk to the optimized medium for protease production was higher than the original medium. In some studies, soy bean and rice bran were also used to increase the protease yield thus, proves that the natural inducers improves protease yield [28], [29].

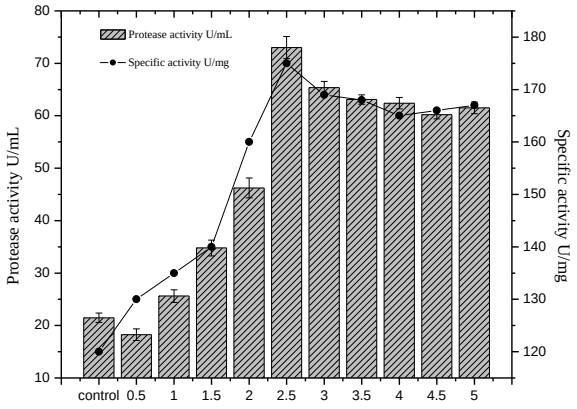

FIG 3: EFFECT OF CONCENTRATION OF RED GRAM HUSK FOR PROTEASE PRODUCTION BY *Streptomyces* sp. LCJ17A

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Experimental design optimization and protease production:

The activities of protease production, main effect, interaction effect and squared effect (nonlinear) of three factors, glucose, casein and red gram husk, at different concentrations were represented in three dimensional response plot (Fig.4-6). Glucose and casein showed increased production of protease. When the red gram husk was added to the medium the production of protease was slightly increased but not to the level of glucose and casein. The interaction effect of glucose (A) and casein (B) was found to be highly significant (P-value of AB = 0.001), implying that glucose and casein were essential for protease production. The optimal concentrations of glucose (A) and casein (B) for *Streptomyces* sp. LCJ17A were found to be 3.5 g/L for glucose and 0.5 g/L for casein in response surface plot. The prediction of the model was validated by additional experiments in triplicates at shake flask level using the optimized medium. These experiments yielded maximum of 88.2±0.9 U/mL enzyme, which was three times higher than the un-optimized medium. In other studies, researchers found improved production by optimizing media using statistical RSM methods for protease production using *Bacillus* sp. RGR-14 [30].

The results of central composite design (CCD) experiments for studying the effects of three independent variables, viz., glucose and casein and red gram husk, on protease production are presented in TABLE III along with the mean predicted and observed response. The regression equation obtained after the analysis of variance (ANOVA) indicated the R^2 value of 0.8977 (a value of $R^2 > 0.75$ indicates the aptness of the model), which ensured a satisfactory adjustment of the quadratic model to the experimental data.

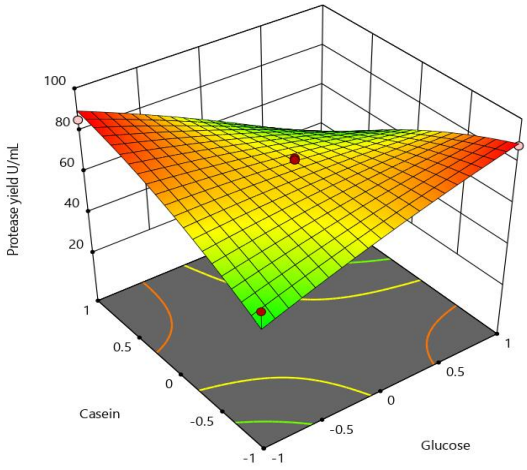

FIG 4: THREE DIMENSIONAL GRAPH OF THE INTERACTION BETWEEN GLUCOSE AND CASEIN ON THE PROTEASE PRODUCTION BY *Streptomyces* sp. LCJ17A

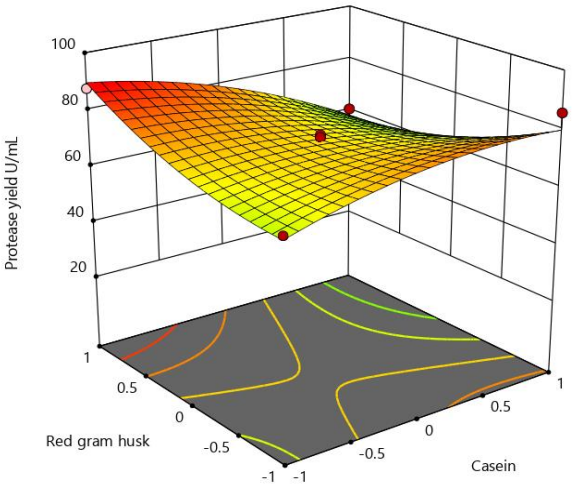

FIG 5: THREE DIMENSIONAL GRAPH OF THE INTERACTION BETWEEN CASEIN AND RED GRAM HUSK ON THE PROTEASE PRODUCTION BY *Streptomyces* sp. LCJ17A

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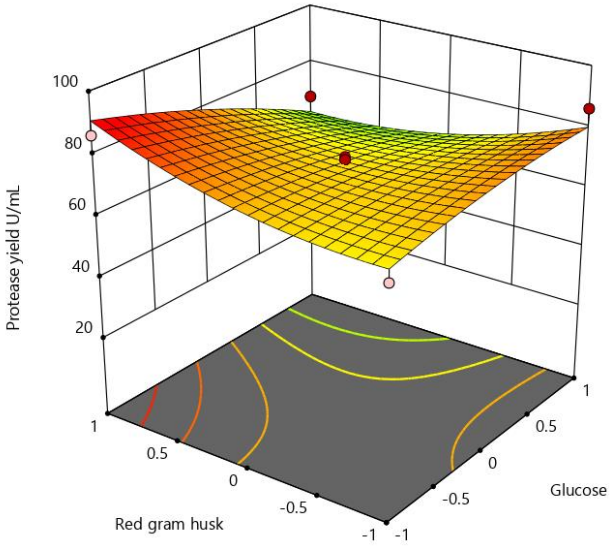

FIG 6: THREE DIMENSIONAL GRAPH OF THE INTERACTION BETWEEN GLUCOSE AND RED GRAM HUSK ON THE PROTEASE PRODUCTION BY *Streptomyces* sp. LCJ17A

An adequate precision of 12.5014 indicates an adequate signal as it measures the signal-to noise ratio. The coefficients of the regression equation were calculated using Design Expert, and the following regression equation was obtained.

$$Y = 73.44 - 5.12X_1 - 4.67X_2 + 0.2375X_3 - 2.09X_1^2 - 4.68X_2^2$$

$$+ 5.08X_3^2 - 22.62X_1X_2 - 11.01X_2X_3 - 9.09X_1X_3$$

With Y, protease production (response); X_1, Glucose; X_2, Casein and X_3, Red gram husk for *Streptomyces* sp. LCJ17A

To understand the medium components interaction and the optimum concentration of each component required for maximum production of protease, three dimensional response surface curves were plotted. The adjusted R^2, which is more suited for comparing models with different numbers of independent variables, was 0.7953. The coefficient of variance was 8.65 (TABLE IV). The predicted sum of squares (PRESS), which is a measure how a particular model fits each point in the design was 3140.25. The adequate precision value, which measures the 'signal to noise ratio' was found to be 12.5014, which indicates an adequate signal. A ratio >4 is desirable. This model can be used to navigate the design space.

TABLE III: RESULTS OF CCD USING THREE INDEPENDENT VARIABLES AND SIX CENTER POINTS SHOWING OBSERVED RESPONSE

	Glucose	Casein	Red gram husk	Response 1
Run	X1	X2	X3	R1
1	1	1	0	20.91
2	0	0	1	86.98
3	0	-1	0	80.01
4	1	0	0	75.24
5	-1	1	0	85.58
6	0	1	0	75.56
7	-1	0	1	86.21
8	0	0	0	75.04
9	0	1	0	62.53
10	1	0	1	67.86
11	-1	0	-1	68.15
12	0	-1	1	87.69
13	0	1	1	60.73
14	0	-1	0	60.71

15	-1	0	0	88.20
16	0	0	1	70.08
17	0	0	0	74.04
18	0	0	0	74.04
19	0	-1	-1	68.67
20	0	0	-1	60.89
21	-1	-1	0	62.12
22	0	0	0	70.04
23	0	0	-1	87.08
24	1	0	0	60.69
25	0	1	-1	85.76
26	-1	0	0	69.89
27	0	0	0	74.04
28	1	-1	0	87.92
29	1	0	-1	86.15

TABLE IV: REGRESSION ANALYSIS

ANOVA	
Std. Dev.	6.30
Mean	72.86
Coefficient of variance	8.65
R^2	0.8977
Adjusted R^2	0.7953
Predicted R^2	0.4223
Adeq Precision	12.5014
PRESS	3140.25

IV. CONCLUSION

The present study deals with medium formulation for protease production by *Streptomyces* sp. LCJ17A utilizing red gram husk with optimized concentration of glucose and casein. The strain *Streptomyces* sp. LCJ17A, which was isolated from sediments of mangrove showed higher yield of protease with the optimized concentrations of glucose and casein along with natural inducer red gram husk. There has been very less number of work was carried out from the *Streptomyces* sp. isolated from sediments of mangrove for maximizing protease production for industrial applications. Further studies are still under investigation to fulfill the ultimate aim of maximizing the protease production in higher scales. The results indicate that the use of glucose, casein and red gram husk as rich substrates for maximum protease yield and the enzyme activity from the cheaper source enhances the stability of the protease enzyme for industrial applications. However, future studies on purification and characterization of *Streptomyces* sp. LCJ17A, would help to extent its applications in various industrial and biotechnological aspects.

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