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MEDIUM FORMULATION AND OPTIMIZATION OF CULTURE CONDITIONS TO MAXIMIZE PROTEASE PRODUCTION BY *NOCARDIA* SP. LCJ15A

Dhavala Swarna, J. Joel Gnanadoss*

Microbial and Environmental Biotechnology Research Unit, Department of Plant Biology and Biotechnology, Loyola College, Chennai, Tamil Nadu, India

**Corresponding author: joelgna@gmail.com*

ABSTRACT

The objective of this study was to optimize the production medium for *Nocardia* sp. LCJ15A, isolated from the soil samples of Pichavaram mangroves, Tamil Nadu which showed high yields of protease activity. The isolate *Nocardia* sp. LCJ15A was studied in 6 separate basal media for selection of the best medium. Subsequently protease production by *Nocardia* sp. LCJ15A was further optimized by submerged fermentation. Various physical and nutritional parameters were optimized for increasing the production of protease. Optimized parameters included carbon, nitrogen sources, inducers and their concentration, pH and temperature. Plackett-Burman design assisted in finding the important parameters which showed a strong influence on protease production by *Nocardia* sp. LCJ15A. Maltose (A), casein (B), pH (C) and temperature (D) were selected as model factor for the design. A full factorial design was used to study the effects of carbon and nitrogen sources in protease production by *Nocardia* sp. LCJ15A. The 4 main factors were evaluated by one factor at a time (OFAT) method. This statistical optimization method led to production of 350.99±1.8 U/mL of protease which was 3.5 fold greater when compared to unoptimized medium. Analysis of variance (ANOVA) revealed a high coefficient of determination (R_2) value of 0.9154, confirming a reasonable adjustment of the quadratic model with the experimental data. Production of improved protease enzyme yields from *Nocardia* sp. LCJ15A maximizes its applicability in various industries.

Keywords: *Nocardia* sp. LCJ15A, Optimization, Protease, Actinomycetes, Response Surface Methodology and Plackett–Burman design.

1. INTRODUCTION

Proteases are considered as the most vital industrial enzymes that account for 60% of the overall enzyme market [1]. They find application in detergent, dairy, meat, protein, photographic, brewing and leather industries [2]. Increasing demand of proteases had led researchers to search for promising sources of proteases [3]. Bacterial enzymes are the cheapest sources that are widely used in food, textile, and beverage industries along with waste treatment processes [4]. Proteases from many other bacteria have been extensively characterised and studied because of their noteworthy part in cellular metabolic process and also in industrial community, but the proteases from actinomycetes did not gain much attention [5]. Large scale production of proteases in industries is mainly carried out under submerged fermentation, where enzyme recovery is a very simple process [6]. Under submerged fermentation, several conditions have been described to stimulate proteolytic enzyme production. Media components for enhanced protease production have been noticed to be different for each species when studied in submerged fermentation. Therefore, media components like inducers, carbon and nitrogen source besides physical aspects like temperature, growth time, pH, inoculum size and aeration need to be optimized to substantiate a successful fermentation method for enzyme production [7]. Development of appropriate medium and cultural conditions is necessary to gain the optimum enzyme yield. Statistical optimization enables a big experimental domain to be screened rapidly, along with signifying the importance of components [8]. Response surface methodology (RSM) is usually used for optimization studies in several biotechnological and industrial processes [9]. RSM is useful for designing, enhancing and optimizing methods, for constructing a list of designs to offer suitable response indications. [10]. Discovering new species having higher protease activity with new traits will be benefitting the

enzyme industry; these new species may be used in different applications [11]. Achieving the basic industrial objective of producing enzymes using a novel isolate is likely if the strain is studied for its growth and enzyme production aspects [12]. This can be accomplished by optimizing several parameters. Therefore, the purpose of this work was to formulate a suitable medium, optimize various nutritional and physical parameters and to study response surface methodology for protease production by *Nocardia* sp. LCJ15A.

2. MATERIAL AND METHODS

2.1. Isolation and screening of *Nocardia* sp. LCJ15A for protease activity

The Actinomycete isolate *Nocardia* sp. LCJ15A was isolated from Pichavaram mangrove forest soil using Humic acid vitamin agar (HVA) medium. *Nocardia* sp. LCJ15A showed high proteolytic activity on skim milk agar medium. Genomic DNA was isolated and PCR amplification was done by universal primers. The cultures were then identified by 16S rRNA sequence. The nucleotide sequence of the 16S rRNA thus obtained was subjected to BLAST analysis with the NCBI database, and phylogenetic tree was constructed using the MEGA 6 software. The culture was maintained in ISP-2 slants and also in glycerol stock and was stored at 4°C.

2.2. Selection of liquid medium for protease production

Liquid medium is the main criteria for production of protease by submerged fermentation. Keeping this in view protease production was studied in six different basal media: Starch Casein Broth [13] as Medium 1, Glycerol-peptone salt broth [14] as Medium 2, Protease production broth [15] as Medium 3, Malt yeast extract broth [16] as Medium 4, Gelatine broth [17] as Medium 5 and Modified Nutrient Glucose Broth [18] as Medium 6. All the flasks containing different media were complemented with a pinch of Nalidixic acid, Nystatin and Actidione to avoid bacterial and fungal contamination. 100 ml medium was prepared and autoclaved at 121°C for 15 mins. Under sterile conditions four mycelial discs of *Nocardia* sp. LCJ15A were inoculated into the flasks and incubated under shaking conditions at 120 rpm at 30°C. At every 2 days interval 10 ml of culture filtrate was taken and centrifuged at 3500 rpm for 15 mins to remove mycelia and medium debris and readings were taken at 660 nm.

2.3. Time course of protease production

Time course of protease production was studied using growth curve obtained by inoculating the culture *Nocardia sp*. LCJ15A in 100 ml protease production broth. Readings were taken on alternative days at 660 nm and the experiment was carried out for 16 days.

2.4. Assay for Protease

Keay and Wildi method [19] was followed to study protease activity in the culture filtrate. The assay mixture contained 200 µl of enzyme extract to which 500 µl of 1% casein was added. Subsequently 300 µl of 0.2 M phosphate buffer (pH 7) was added and allowed to react for 10 min at 37°C. Further 1ml of 10% TCA (Trichloroacetic acid) was added to stop the reaction and then centrifuged at 3000 rpm for 15 mins. An aliquot of 1ml of supernatant was mixed with 5 ml of 0.4 M sodium carbonate (Na_2CO_3) followed by the addition of 1ml of Folin-Ciocalteu's phenol solution. After 30 mins of incubation, the absorbance of the supernatant was spectrophotometrically measured at the wavelength of 660 nm [20]. One unit of the protease activity (U) is defined as the amount of enzyme liberating 1µg of tyrosine per ml at 37°C for 30 minutes [21]. Tyrosine 10-100 µg was used as standard to calculate enzyme units.

2.5. Determination of protein

Protein concentration in the supernatant was studied by Lowry *et al.,* method [22]. Specific activity was measured as the ratio between enzyme activity and the protein concentration. Specific protease activity was calculated and expressed as units/mg protein.

2.6. Optimization studies for protease production using submerged fermentation

The standard method of medium optimization includes varying one independent variable like carbon, nitrogen and inducer while keeping other variables constant [23]. It is essential to study the process conditions and optimization of fermentation medium to maximize the profits from fermentation process. In deciding the cost of enzyme production, medium optimization is an important parameter [24]. Carbon and nitrogen sources have a major role in the metabolism of essential nutrients required for organism growth in the medium. Therefore, in order to achieve optimum production of the desired enzyme it is important to optimize the carbon and nitrogen sources along with inducers. The conventional "one-factor-at-a-time" method was used to optimize physical and nutritional parameters by varying one

parameter at a time while fixing the other parameters as constant.

2.7. Effect of carbon source and their concentration on protease production in Protease Production Broth (PPB)

The influence of different carbon sources on the production of protease by *Nocardia* sp. LCJ15A was studied. Sucrose, starch, fructose, lactose, glycerol maltose and mannitol were the carbon sources used. Glucose the original carbon source of the medium was substituted by different carbon sources at 5 g/L concentration, in separate sets of experiments while the actual medium was used as the control. After the selection of best carbon source, the concentration of the best carbon source was further optimized in varying concentrations of 0.5 to 5 g/L. Protease and protein activity were determined at 660 nm.

2.8. Effect of nitrogen source and their concentration on protease production in Protease Production Broth (PPB)

The influence of different nitrogen sources on protease production by *Nocardia* sp. LCJ15A was studied using both inorganic and organic nitrogen sources. Organic nitrogen sources included urea and casein and inorganic sources included ammonium nitrate and sodium nitrate was studied. Peptone and yeast extract, the original nitrogen source of the medium was substituted by different nitrogen sources at 5 g/L concentration in separate sets of experiments. After selecting the best nitrogen source, the concentration of the best nitrogen source was further optimized in varying concentrations like 0.5 to 5 g/L.

2.9. Effect of inducers and their concentration on protease production in Protease Production Broth (PPB)

The influence of different natural and chemical inducers on the production of protease by *Nocardia* sp. LCJ15A was studied. Protease production broth was supplemented with natural inducers like green gram husk, red gram husk, black gram husk, sesame oilcake and groundnut oilcake oil at 3 g/L concentration in separate sets of experiments. After selecting the best natural inducer, the concentration of the best natural inducer for *Nocardia* sp. LCJ15A was further optimized in varying concentrations like 0.5 to 3 g/L. In the same way the influence of different chemical inducers on the production of protease by *Nocardia* sp. LCJ15A was studied. Protease production broth was supplemented with chemical inducers BSA, casein and gelatine at 3 g/L concentration. After selecting the best chemical inducer, the concentration of the best chemical inducer was further optimized in varying concentrations like 0.5 to 3 g/L.

2.10. Effect of pH on protease production in Protease Production Broth (PPB)

The influence of initial medium pH on protease production by *Nocardia* sp. LCJ15A was evaluated. The actual pH of PPB was 7 and various pH ranges were adjusted from 4 to 10 using 0.1N HCL and 0.1N NaOH. The protease activity was studied on alternate days.

2.11. Effect of temperature on protease production in Protease Production Broth (PPB)

The influence of different incubation temperatures ranging from 30°C to 40°C on the protease production by the isolate *Nocardia* sp. LCJ15A was studied in Protease Production Broth (PPB).

2.12. Medium optimization by statistical analysis

2.12.1. Experimental design for optimizing protease production by Nocardia sp. LCJ15A

The statistical optimization of medium components for protease production was studied in two stages.

i) Plackett-Burman Design (PBD)

The Plackett-Burman Design (PBD) is a helpful means for screening the important factors between several variables [25]. Plackett-Burman Designs are used for screening the medium components that remarkably influenced protease production. Each variable was usually verified at two levels, low (-) and high (+). The name, code and level of variable of Plackett-Burman experimental Design are represented in Table 1. The experimental runs were studied in triplicates and the average value was considered as the response. The Pareto chart exhibited the variables showing highest positive effect and were considered to have greater impact on protease production and was chosen for further optimization using CCD and RSM for maximizing protease production. Plackett-Burman Design follows the first-order polynomial model (Eq. 1) and is a two-level fractional design, $Y = \beta_0 + \sum \beta_n X_n$ (1) Where Y is the predicted response, β_0 is the value of the model intercept, β_n is the linear coefficient and X_n is the level of independent variable

Table 1: Medium components for protease production by *Nocardia* sp. LCJ15A using Plackett-Burman Design (PBD)

Variables	Low level (-)	High level
Maltose (g)	0.5	0.2
Casein (g)	0.1	1
pH	0	0.5
Temperature	0	0.4
Green gram husk	0	0.8
BSA	1	0.6

ii) Response Surface Methodology (RSM)-Central Composite Design (CCD)

The core principle of RSM is to employ a series of designed experiments to achieve an ideal response. The type of the response surface plot in the optimum area is studied by 2 ways. A series of experimental design Face Centred Central Composite Design (FCCCD) was the first step followed by RSM for enhancing the alkaline protease production. In the experimental design each individual factor was studied at 3 different levels from -1, 0 and + 1. A set of 29 experiments were studied involving 4 main factors. All the main variables were taken at a central coded value which is equal to zero. After the experiment is completed, the average of the maximum protease yield was considered as the response or dependent variable (*y*). Multiple regression method was applied to fit in the second ordered polynomial equation into the data. This helped in finding an experimental model that related to the response determined by the independent variables of the experiment. Protease production by *Nocardia* sp. LCJ15A under submerged fermentation was controlled by 4 important factors, maltose (A), casein (B), pH (C) and temperature (D). A response surface methodology using a two-step experimental design was studied taking the above factors in consideration. The following quadratic equation was used to calculate the result for a four factor system.

$$Y= \beta_0 +\beta_1X_1 +\beta_2X_2 +\beta_3X_3 +\beta_4X_4+\beta_{11}X_1^2 +\beta_{22}X_2^2 +\beta_{33}X_3+\beta_{44}X_4^2+\beta_{12}X_1X_2 +\beta_{13}X_1X_3+\beta_{14}X_1X_4 +\beta_{23}X_2X_3 +\beta_{24}X_2X_4 +\beta_{34}X_3X_4 \quad ....(2)$$

where, *Y,* predicted response; β_0 intercept; β_1, β_2, β_3, β_4 linear coefficients; β_{11}, β_{22}, β_{33}, β_{44} squared coefficients; β_{12}, β_{13}, β_{14}, β_{23}, β_{24}, β_{34} interaction coefficients [26].

2.13. Validation of protease production under modified and original condition

The validation of the optimized factors was studied under conditions predicted by the model by the optimized values of the process parameters. The protease production by *Nocardia* sp. LCJ15A was compared between the modified and original production media. 100 mL of the modified and original medium were inoculated with *Nocardia* sp. LCJ15A culture discs (4 mm) and incubated at 30°C. The protease activity was then assayed on alternate days.

3. RESULTS & DISCUSSION

3.1. Isolation and screening of *Nocardia* sp. LCJ15A for protease activity

Nocardia sp. LCJ15A, a novel actinomycete was isolated from the soil sample of Pichavaram mangrove forest. The culture showed high enzyme activity and was characterized using molecular techniques. Genomic DNA was isolated and amplified by PCR using the universal primers. DNA was sequenced using Sanger's dideoxy method using big dye terminator v3.1. The sequence was compared with those in the National Centre for Biotechnology Information (NCBI) nucleotide sequence database using Basic Local Alignment Search Tool (BLAST). Subsequently the sequence was submitted in NCBI using 16S rRNA submission tool through BankIt and accession number KU921111 was obtained. The phylogeny was constructed using MEGA 6.0. software (Fig. 1) and showed homology with *Nocardia Africana,* when compared with the most closely related sequences in a NCBI database.

3.2. Selection of liquid medium for protease production

The protease production was considerably different in all the 6 media tested. Enzyme production of selected isolates was monitored over a period of 12 days. Protease and protein activity were measured during alternative days. Among the six different basal media, Protease production broth i.e., Medium 3 favoured optimal enzyme production of *Nocardia* sp. LCJ15A. Increased enzyme activity was noted on 8th day and on 10th day a simultaneous decline in specific protease activity was observed. In Medium 3, a maximum protease activity of 122.98 U/mL was recorded on the day of incubation in *Nocardia* sp. LCJ15A. The results also proved that other media also showed enhanced protease production in *Nocardia* sp. LCJ15A culture but significantly less compared to Protease Production Broth (PPB).

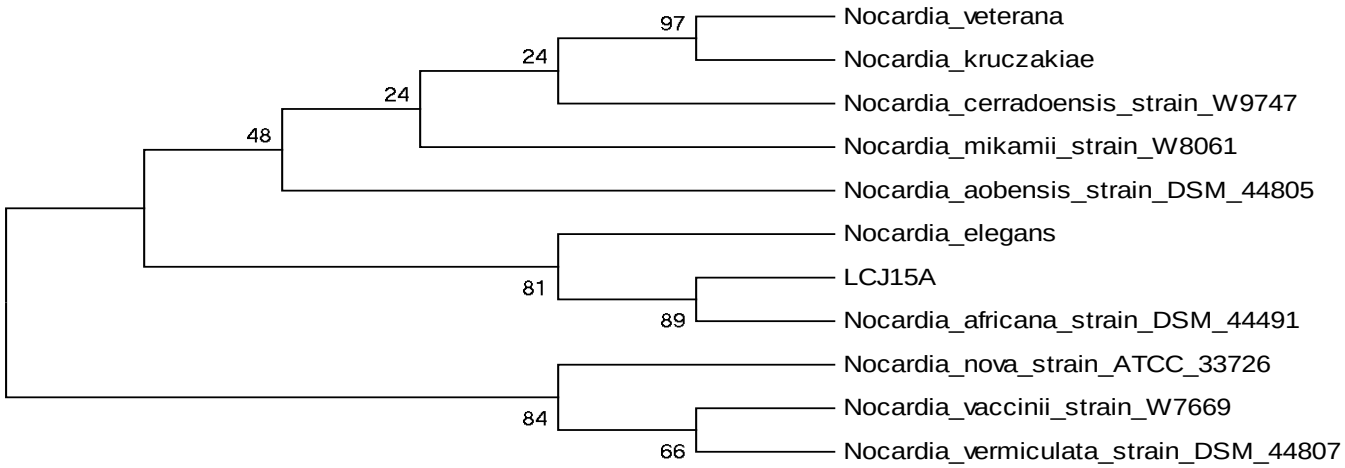

Fig. 1: Neighbor-Joining tree of the ITS region of strains LCJ15A and with higher similarity from the Genbank selected from the results of a BLAST search

3.3. Time course of protease production

The growth pattern and protease production by *Nocardia* sp. LCJ15A was studied. The protease activity was significantly higher on the 8th day and increase in biomass was observed on the 8th day. Subsequently on the 10th day, a decline in protease activity was noted with an increase in biomass. Nochure and Roberts reported that the actual cause for increased incubation time and declined protease activity could be due to exhaustion of nutrients in the production medium [27]. This could be attributable to altered physiology that led to secretary machinery inactivation of the enzymes. In a similar study by Oyeleke, maximum protease production was reported on 6th day of incubation [28].

3.4. Effect of carbon source and their concentration on protease production in Protease Production Broth (PPB)

The carbon source used in the medium certainly has a major effect on enzyme production. The results showed that *Nocardia* sp. LCJ15A was able to grow in the various tested carbon sources and showed an increased protease production in maltose with 83.64 U/mL in PPB medium as shown in table 2. Generally complex sources of carbon and nitrogen are needed for the production of proteases, but requirements for particular sources of carbon and nitrogen differ from organism to organism [29]. Maltose favoured the protease production which could be because of the organism's capacity to breakdown disaccharides into simple sugars and that it was readily available for the metabolism and growth of the organism. The concentration of maltose ranging from 0.5 to 5.0 g/L was also studied in PPB. Optimal protease activity of 148.5 U/mL was recorded at 3.0 g/L of maltose is shown in Fig 2.

3.5. Effect of nitrogen source on protease production in Protease Production Broth (PPB)

Different organic and inorganic nitrogen sources were employed to study their influence on protease production. All the nitrogen sources studied supported the organism's growth and also enhanced protease production compared to the control. Nevertheless, the highest enzyme output was for casein with an activity of 157.66 U/mL. Other nitrogen sources, in combination with yeast extract or either alone, showed a reduced enzyme activity.

3.6. Effect of inducers on protease production in Protease Production Broth (PPB)

The influence of several natural inducers like groundnut oilcake, sesame oilcake, green gram husk, black gram husk and red gram husk on the production of protease was studied. Addition of natural inducer (green gram husk) to the medium favoured optimum protease activity of 183.11 U/mL. Similarly, the influence of different concentrations of the best natural inducer, green gram husk was studied in varying concentrations from 0.5 to 3.0 g/L. Maximum protease activity of 185.51 U/mL was observed at 2.5 g/L of green gram husk as shown in Fig 4. Subsequently the influence of chemical inducers such as BSA, gelatine and casein on the production of protease in PPB was studied. The

yield of protease with addition of BSA was higher with an enzyme activity of 158.12 U/mL. Similarly the influence of different concentrations of chemical inducer, BSA was studied in varying concentrations from 0.5 to 3.0 g/L in PPB. Enhanced protease activity of 140.82 U/mL was recorded at 3.0 g/L of BSA as shown in Fig 5.

Table 2: Effect of different carbon sources, nitrogen sources, natural and chemical inducers in the culture medium for protease production by *Nocardia* sp. LCJ15A

Nutrient Source	Protease yield
Carbon source (5 g/L)	
Sucrose	22.01±0.4
Starch	39.83±1.2
Fructose	31.29±0.8
Lactose	34.61±0.8
Glycerol	25.33±1.2
Maltose	83.64±1.4
Mannitol	24.99±1.6
Nitrogen source (5 g/L)	
Urea	124.6±2.5
Casein	157.66±1.2
Ammonium nitrate	132.89±1.2
Sodium nitrate	101.06±4.7
Natural Inducer (3 g/L)	
Green Gram husk	183.11 ±1.4
Red Gram husk	168.32± 0.7
Black Gram husk	174.94± 1.9
Sesame oilcake	157.55± 1.3
Groundnut oilcake	144.37± 2.7
Chemical Inducer (3 g/L)	
BSA	130.51±1.8
Casein	158.12± 0.9
Gelatine	107.59± 0.8

Therefore protease production was more with casein when compared to inorganic sources in PPB. Further casein concentration was also optimized in varying concentrations like 1 to 5 g/L and optimal protease activity of 167.97 U/mL was noticed at 2.0 g/L of casein is shown in Fig 3. In a similar study by Bajaj in 2011, an alkali-tolerant *Streptomyces* sp. DP2 utilized soybean meal as best nitrogen source for protease production [30]. A few studies have reported favourable protease production when tested with complex nitrogen sources [31].

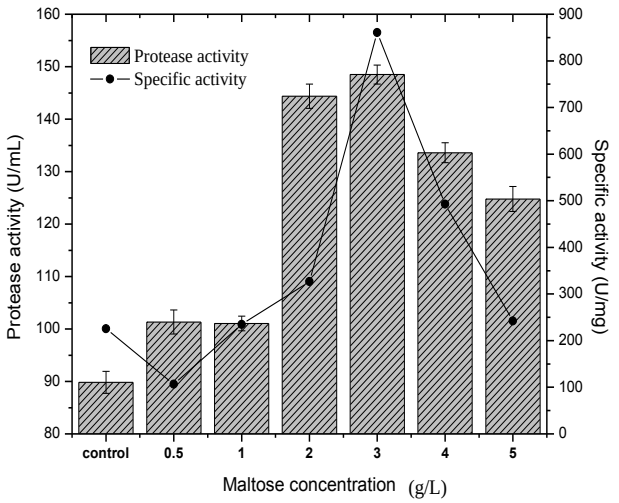

Fig. 2: Effect of concentration of Maltose on protease production by *Nocardia* sp. LCJ15A in Protease Production Broth on 8th day (peak day)

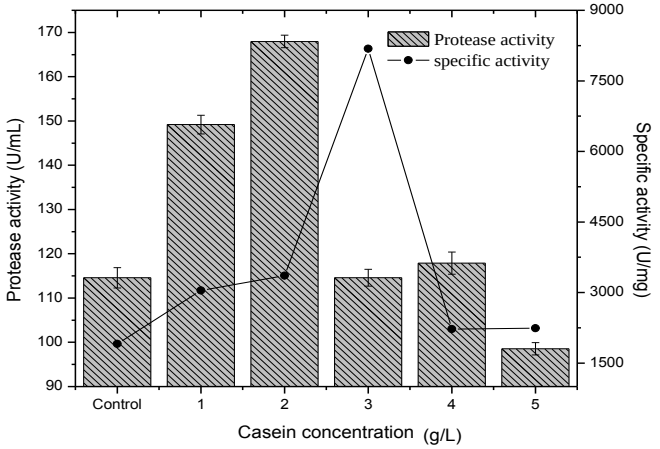

Fig. 3: Effect of Casein concentration on protease production by *Nocardia* sp. LCJ15A in Protease Production Broth on 8th day (peak day)

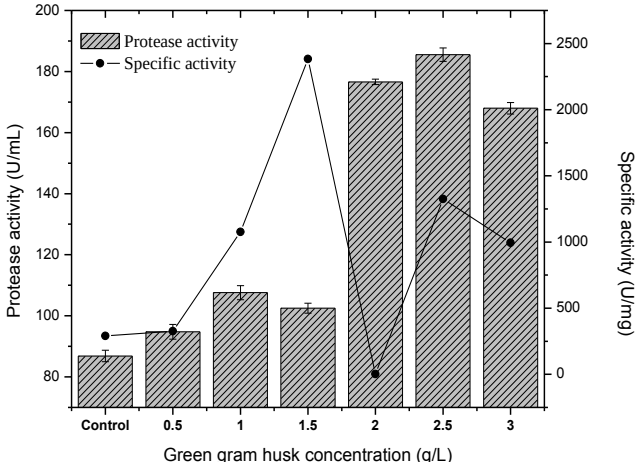

Fig. 4: Effect of concentration of Green gram husk on protease production by *Nocardia* sp. LCJ15A in Protease Production Broth on 8th day (peak day)

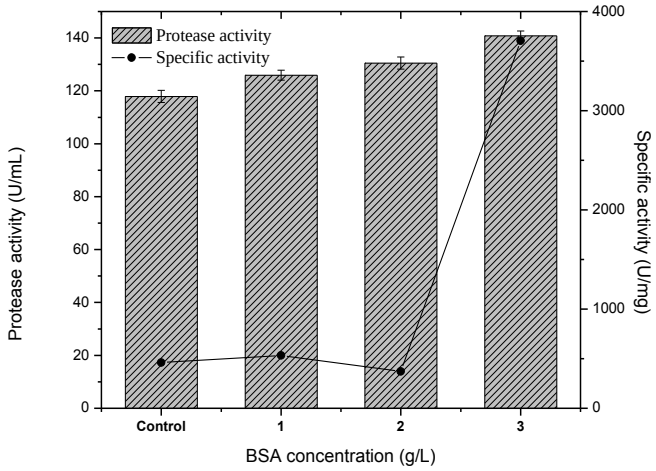

Fig. 5: Effect of BSA concentration on protease production by *Nocardia* sp. LCJ15A in Protease Production Broth on 8th day (peak day)

3.7. Effect of pH on protease production in Protease Production Broth (PPB)

The physical parameters are considered as important for enzyme production. The present study showed that favourable protease production was noticed at pH 10 with an activity of 120.88 U/mL. A further rise in pH above 10 caused a decline in protease production. Since pH 10 favoured the protease production the enzyme was proved to be alkaline in nature. The pH of the culture medium has a significant impact on several enzymatic reactions and the transport of different components across cell membranes, promote enzyme production and cell growth [32].

3.8. Effect of temperature on protease production in Protease Production Broth (PPB)

Mostly the suitable incubation temperature varies significantly from one strain to another. Enhanced protease production was noted at a temperature of 32°C for growth and protease production, showing 243.87 U/mL enzyme activity. Temperatures higher than 32°C caused a reduction in the protease production. Therefore a temperature condition ranging from 30°C to 32°C was considered optimal for protease production. This study is in agreement with the findings of Saravana Kumar (2010) conclusions that the medium composition and some physical factors like the pH, fermentation period and temperature favoured the extracellular production of protease [33].

3.9. Optimizing protease production by *Nocardia* sp. LCJ15A using Response surface methodology

To analyse the interactive effects of different nutritional and physical factors on protease activity by *Nocardia* sp. LCJ15A, the statistical design method with RSM was employed. Fig. 6 shows the Pareto chart of *Nocardia* sp. LCJ15A which illustrates the significant factors for enzyme activity. In addition, it directly shows that the most important factors influencing protease production are maltose, pH and temperature. Table 3 shows the Plackett-Burman Design matrix, the observed and predicted values for screening of important variables for-protease production by *Nocardia* sp. LCJ15A.

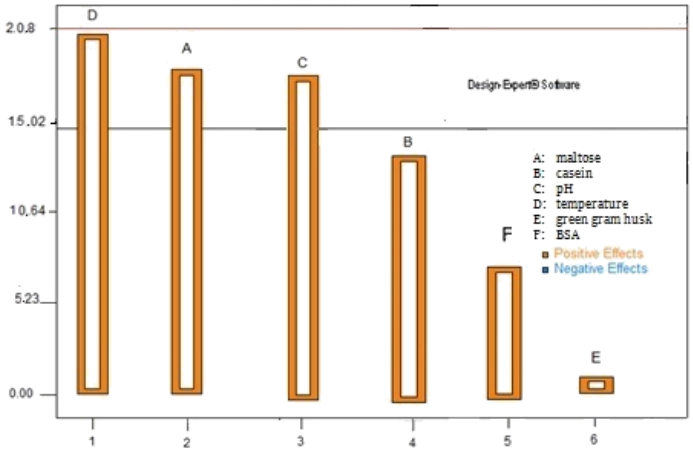

Fig. 6: Pareto chart showing the effect of six factors on protease production by *Nocardia* sp. LCJ15A

The input variables which have the most impact on the system's final response were established from the one at a time strategy and four factors interaction (two nutritional and two physical) namely, Maltose (A), Casein (B), pH (C) and temperature (D) was observed by RSM following the FCCCD. Figure 7 and 8, shows the three dimensional surface plot of interaction between the 4 variables which also represents the behaviour of protease production, squared effect, interaction effect and main effect of the 4 variables. From the 3D plot it is evident that maltose, casein, pH and temperature helped in maximizing protease production to a greater extent. The actinomycete strain, *Nocardia* sp. LCJ15A showed the maximum predicted enzyme production that can be obtained using the optimum concentrations of medium components at 95% confidence level was 85.93 U/mL. Additional experiments in duplicates, at shake-flask level were

conducted by optimized medium to verify the model prediction. These experiments showed 350.99±1.8 U/mL enzyme activity, which was 3.5 times more than the un-optimized medium. It is also important to note that the interaction outcome of the components was significant. The maximum protease production was observed after 7 days when the levels of maltose and casein were at their central value of 3 and 2.5 g/L respectively. For predicting the model point, a second order polynomial function was fitted to the experimental results of FCCCD. However, the model equation predicted that the enzyme production was subject to catabolite suppression by maltose and favourable protease production could be achieved using less amounts of carbon sources. Enzyme production was inhibited at higher levels of pH and temperature. Evaluation of all the plots indicated where optimum conditions exist within the experimental area covered, or in what way further experiments are required to attain better results. The results predicted by model equation from FCCCD showed that a blend of high concentrations of casein, central value of maltose would favour protease production in case of *Nocardia* sp. LCJ15A.

Table 3: Plackett-Burman Design matrix, their observed and predicted values for screening of important variables for-protease production by *Nocardia* sp. LCJ15A

Run	Maltose (g)	Casein (g)	pH	Temp	Greeen gram husk (g)	BSA(g)	protease activity (U/mL)	
							Observed value	Predicted value
1	3.0	1.5	4.0	30	1.5	1.0	58.63	49.77
2	2.5	1.0	6.5	34	0.5	0.5	42.64	43.60
3	2.0	2.5	7.0	36	2.0	2.5	30.71	21.10
4	2.5	2.0	10.0	26	2.5	2.5	39.58	34.74
5	1.5	3.0	10.5	28	1.0	2.5	47.68	41.93
6	1.0	1.5	11.0	32	2.5	2.0	78.88	82.34
7	1.5	1.0	8.0	36	2.0	2.5	68.41	62.47
8	0.5	1.0	8.5	38	3.0	1.5	58.88	53.05
9	2.0	1.0	4.5	40	1.5	1.5	45.10	54.53
10	2.5	0.5	5.0	38	0.5	1.0	21.13	43.44
11	0.5	2.5	4.5	24	2.5	1.0	30.99	17.27
12	1.0	2.5	3.5	30	2.5	2.5	40.96	29.88
13	1.5	0.5	6.0	32	0.5	0.5	45.32	32.98
14	2.0	3.0	5.5	36	2.0	0.5	38.77	42.11

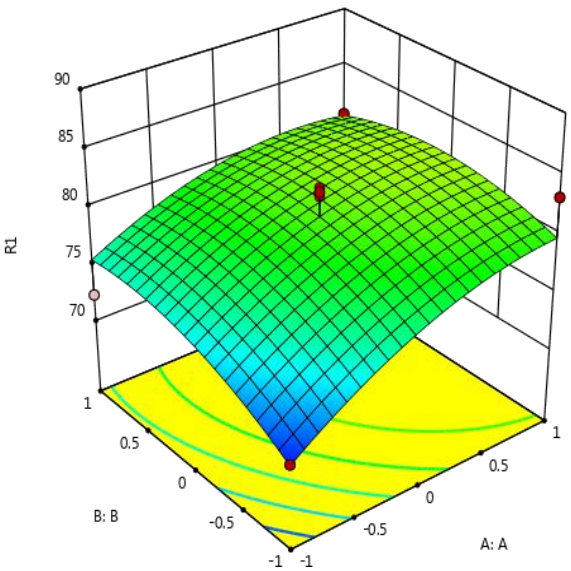

Fig. 7: Three dimensional graph of the interaction between maltose and casein

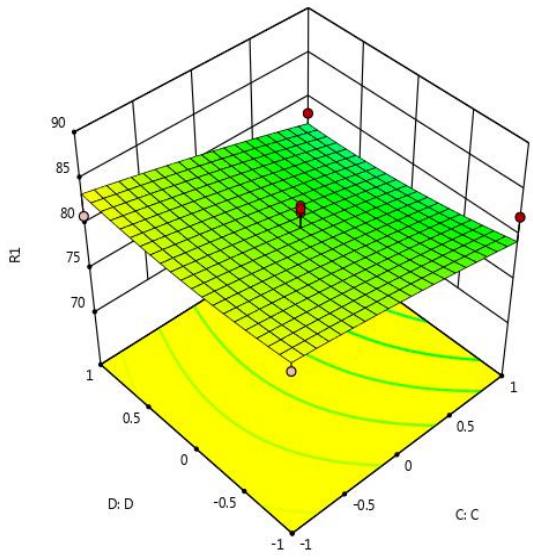

Fig. 8: Three dimensional graph of the interaction between pH and temperature

FCCCD results for the 4 factors studied is given in Table 4. R^2 value of 0.9154 (a value of $R^2 > 0.8057$ indicates the aptness of the model), indicated that 90% of the differences in the response could be described by the model. An adequate precision of 12.48 indicates an adequate signal since it calculates the signal-to noise ratio. The coefficient of variance was 4.27%. Design Expert software was used for calculating the coefficients of the regression equation, and the following equation was obtained.

$$Y = 85.93-0.94X_1-0.584X_2-0.612X_3-1.29X_1X_2-1.68X_1X_3+ 0.6651 X_2X_3- 0.51 X_1X_4- 2.19 X_1^2-3.81X_2^2-3.19 X_3^2$$

with Y, protease production (response); X_1, maltose; X_2, casein and X_3, pH and X_4, temperature for *Nocardia* sp. LCJ15A. The 3D response surface plots are drawn to understand the interactions between the model variables and the optimum value required to maximize protease production for each variable. Almost all interactions have produced a 'nearly spherical' variance function that suggests a well-defined optimum for protease production. The present investigation validates the use of a statistical approach for determining the interaction between four main variables using RSM for alkaline protease production by CCD.

The results predicted after statistical design showed a higher correlation with those obtained after conventional methods. This study was the first in a series of studies emphasizing a scale up approach for protease production by *Nocardia* sp. LCJ15A utilizing the optimized concentration of maltose and casein besides pH and temperature. For enzyme production, submerged fermentation is the favoured approach by manufacturers of commercial enzyme production [34]. Medium components are optimized to ensure a balance between the different medium components and thereby reducing the quantity of unused components [35]. The present study revealed that organic nitrogen source showed better protease production compared to inorganic nitrogen sources. This enhanced production with organic nitrogen sources can be associated to their capability to induce enzyme activity to high levels. Enshasy (2008) emphasized that for the enhancement of yield of proteases, the optimization of the production medium and the physical conditions are to be considered to establish a commercially efficient technology [36]. Better fermentation conditions will enhance higher production of proteases. Extracellular protease production in microorganisms usually depends on medium components like carbon and nitrogen sources [37]. Organic nutrients are also less expensive and helps in supplying the necessary minerals and vitamins required for protease production. The cell growth is also further rapid and efficient when organic nitrogen sources are used, since they decrease the number of components that cells would otherwise have to be synthesized [38]. This research's preliminary work centred on recognizing potential variables for enhancing protease activity by Nocardia sp. LCJ15A strain by optimizing various medium elements, using the RSM method. Response Surface Methodology (RSM) was commonly used to test and understand the interactions between the various nutritional and physiological factors. [39]. Response surface methodology is studied by many researchers for optimization of physicochemical parameters from different organisms [40]. There is a huge need to discover new strains that produce industrially important enzymes with novel properties [41]. In this study, an actinomycete strain *Nocardia* sp. LCJ15A was considered for protease production under submerged fermentation. Statistical design is a growing approach to optimize medium components and physical parameters for increased enzyme production. Optimization of enzyme production is studied using various statistical methods, such as Box-Benhken experimental Design [42], Response Surface Methodology [43] and Plackett-Burman experimental Design [44].

For the screening of the most notable factors between several variables, Plackett-Burman Design (PBD) is employed [45]. Plackett-Burman design determines the remarkable factors that play a major role in the enzyme production and CCD analyses the interactions among the factors [46]. A central composite design was adopted for the optimization process. The independent variables selected for this study were Maltose (A), casein (B), pH (C) and temperature (D). Each independent variable in the design matrix was studied at 3 distinct levels (-1, 0, +1). In statistical optimization using response surface methodology the yield of protease was 350.99±1.8 U/mL, the medium composition included maltose 2 g/L, casein 2.5 g/L, pH 10 at 32°C temperature.

In a similar study by Beg, RSM was used for medium optimization for *Bacillus mojavensis* for alkaline protease production from an interaction of various physicochemical factors [47]. Submerged fermentation, is essentially dependent on the state of growth and composition of nutrient medium for the production of commercially important enzymes [48]. The results of

FCCD for studying the influence of 4 independent variables on protease production which clearly shows the mean observed and predicted response, confirm that protease production was enhanced compared to the unoptimized medium. The regression equation value of 0.9154 clearly indicates the aptness of the model since $R^2 > 0.75$. The optimization of protease production by RSM was useful in this particular study, helped in the easy identification of the important factors and interaction among them. Further it also recommended the importance of a range of factors at altered levels.

Table 4: FCCD Results of *Nocardia* sp. LCJ15A using 4 independent variables

RUN	Maltose	Casein	pH	Temp	Observed Value	Predicted value
1	-1	0	0	1	78.31	80.05
2	0	0	-1	0	81.66	83.45
3	0	0	0	-1	84.51	73.11
4	1	0	0	1	65.54	65.21
5	0	0	-1	0	54.02	55.16
6	0	0	0	0	74.10	73.99
7	1	1	0	1	76.19	75.08
8	0	-1	-1	0	69.28	72.16
9	0	1	0	1	85.36	86.66
10	0	0	0	-1	80.67	79.95
11	0	0	-0	1	81.55	82.19
12	0	1	-1	0	73.91	74.44
13	-1	-1	0	0	84.36	84.93
14	0	0	0	1	70.11	68.25
15	0	-1	1	1	78.09	76.55
16	0	1	1	0	84.68	85.11
17	-1	1	0	0	73.25	75.49
18	1	0	0	1	83.94	85.01
19	0	-1	0	1	66.17	65.41
20	-1	0	0	0	73.99	78.38
21	-1	0	1	-1	83.29	83.99
22	1	0	1	1	79.14	81.11
23	1	-1	0	0	82.59	83.13
24	1	0	-1	0	61.97	60.42
25	0	0	1	1	72.66	74.29
26	0	0	1	0	85.09	83.06
27	-1	0	-1	-1	79.63	79.11
28	0	-1	0	1	62.46	64.29
29	0	1	-0	0	74.49	76.68

4. CONCLUSION

The study revealed that effective enzyme production was associated with the growth of *Nocardia* sp. LCJ15A. A combination of Plackett–Burman design and central composite design showed to be effective for understanding the interaction between the variables which influenced protease production. Plackett–Burman design identified that among the six selected factors, temperature significantly influenced protease production. CCD coupled with RSM revealed a 3.5 fold increase in protease production (350.99±1.8 IU/ml) over the control experiments. The present investigation validates the use of a statistical approach for determining the interaction between four main variables using RSM for alkaline protease production by *Nocardia* sp. LCJ15A using central composite design. The results predicted after statistical design showed a higher correlation with those obtained after conventional methods.

Furthermore, the investigation also proved that experimental designs offer an effective and feasible method for the optimization of protease fermentation medium. Validation experiments were performed to confirm the adequacy and the precision of the model. The results also gave a base line for the further study with a large scale fermentation for protease production from *Nocardia* sp. LCJ15A.

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Conflict of interest

The authors declare no conflict of interest.

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