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Research Article

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Optimization of Protease Production by *Streptomyces* SP. LCJ1A Using Response Surface Methodology

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Abstract: Protease is an industrially important enzyme obtained from plants and animals. Production of high amounts of protease at low cost is necessary for its use in different commercial applications. The main objective of this work was to formulate a suitable medium and to optimize various parameters for protease production by *Streptomyces* sp. LCJ1A which was isolated from Pichavaram mangroves, Tamil Nadu, India. The organism was quantitatively and qualitatively screened for protease production. Sequencing of 16S rRNA was done and the sequence was deposited in NCBI and Accession no. KU 870428 was obtained. For optimization, the conventional one-time factor method followed by the Plackett-Burman design was used to optimize various media components. Variables with a significant influence on the production of protease have been identified. Variables such as maltose, peptone, pH and temperature have shown its strong impact on protease production and the ideal concentration of these factors was further investigated by Response Surface Methodology. The Optimum values of tested variables for favourable protease production were: Maltose, peptone, pH and temperature. Further, the interaction effects of maltose and peptone were found to be extremely important suggesting that both components were extremely crucial for protease production. 186.92 U/mL of protease enzyme was obtained in the experimental study which was close to the predicted value of 190.67 U/mL, which could validate the model. Using such optimized variables, the protease production was increased by 10 times when compared with the unoptimized medium. This is the first report on the conventional and statistical optimization of protease production from *Streptomyces* sp. LCJ1A. This study also showed that statistical methods are indeed a better approach for optimization of media, when compared with conventional one-factor-at-a-time method in terms of an enhanced protease yield in a short time

Keywords: Protease, Plackett–Burman Design, Composite Central Design, and Response Surface Methodology, Streptomyces

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1. INTRODUCTION

Microorganisms represent excellent sources of enzymes.[1] The possibility of using actinomycetes for enzyme production, specifically *Streptomyces*, has been recently investigated.[2] *Streptomyces* species emerged as an essential source of various secondary metabolites, enzymes and antibiotics primarily because of their shorter generation time and the ease of environmental and genetic manipulation.[3] Proteases are one of the three most common industrial enzyme classes. Proteases that act on proteins by breaking them down into peptides and amino acids.[4] Traditionally, they hold majority portion of the industrial enzyme market accounting for 60% of total enzyme sales worldwide.[5] Microbial protease has highest commercial value over enzyme obtained from animals and plants.[6] The enzyme has been extensively studied in the last few years because of its potential in various applications.[7-9] Proteases have different applications in several industries such as paper industry, textile industry, biosensors and pharmaceuticals. In submerged fermentation the microorganisms are grown in the liquid medium. Since the contents are immersed in submerged state in the liquid medium, the mass and heat transfer is more effective, and suitable for modelling the process. Though protease is industrially important, its bulk production is not economic due to its high costs and low titres. Medium composition significantly affects product yield, hence a proper fermentation medium is important.[10] Medium optimization is a process where components of medium and culture conditions are changed to get better growth of the organisms for high productivity.[11] It is required to study the optimization of fermentation medium and process conditions to maximize the profits from the fermentation process. In submerged fermentation, factors like different carbon and nitrogen sources and its concentration were studied.[12] Several aspects must be considered during development of a medium of which a very essential one is the final product chosen for fermentation. The present focus is therefore towards the optimization of different media components by conventional or statistical methods. Optimization by conventional methods (one factor at a time) is by varying one parameter at a time while fixing other parameters constant. This method helps to assess the importance of that parameter on the enzyme production. The drawback of conventional methods is, time consuming, need more experimental procedures and cannot exhibit the interactions among the parameters.[13] These drawbacks can be overcome by statistical methods like Response Surface Methodology (RSM) that utilize statistical principles, randomization, replication and duplication.[14] In general, protease production depends on various components of the medium like carbon, nitrogen sources and inducers. Hence, RSM is used to optimize enzyme production statistically when various factors and their interactions are involved in lesser time.[15] Plackett-Burman design determines the important factors that play a major role in enzyme production and Central composite design is used to analyse interactions among the factors. Hence, the aim of this research was to formulate an effective medium, optimize different nutritional and physical variables, and to study *Streptomyces* sp. LCJ1A, using response surface methodology for protease production. The culture medium for *Streptomyces* sp. LCJ1A was systematically optimized by conventional "one factor at a time" method and the important parameters were optimized further by Response Surface Methodology for improving the protease production, which is documented in a few articles. Subsequently to control the level of each significant variable, Central composite design was applied.

2. MATERIALS AND METHODS

2.1 Isolation of Streptomyces sp. LCJ1A

Streptomyces sp. LCJ1A was isolated from soil samples collected from Pichavaram Mangroves, Tamil Nadu, India (latitude 11.42929 and longitude 79.77892). Distinct actinomycetes colonies were isolated and frequently sub-cultured till pure cultures were obtained.[16] The cultures were maintained on starch casein agar plates and incubated at 28-30 °C for 7-10 day.

2.2 Qualitative Screening for Protease Production

Protease production by *Streptomyces* sp. LCJ1A was studied using skim milk agar medium with the following composition in g/L: 3 g of yeast extract, 5 g of NaCl, 10 g of skim milk, 18 g of agar and pH 7.2. After 5 days of incubation, at 32°C, the plates were observed for clear lysing zones around the colonies.[17]

2.3 Molecular Characterization by 16S rRNA Sequencing

Sequencing of 16S rRNA was done for identifying the isolate at the molecular level. PCR amplification of the genomic DNA studied by universal primers 16 S-27 F and 16 S-1492 R.[18]

2.4 Selection of Liquid Medium for Protease Production

Submerged fermentation (SmF) has been characterized as a process studied in an oxygen rich liquid medium with nutrients.[19] Submerged fermentation is a promising option for the competent biomass production and important metabolites. Considering this, protease activity was studied in six separate basal media namely, Protease production broth, Gelatine broth, Starch broth, Glycerol- peptone salt broth, Modified Nutrient Glucose Agar (MNGA) broth and Malt Yeast Extract Broth (MYEB).

2.4.1 Growth media and culture conditions

The production of protease by *Streptomyces* sp. LCJ1A was studied in 100 ml of production medium. Composition of the Starch casein broth (g/L): starch 10 g; casein 3.0 g, KNO_3 2.0 g, NaCl -2.0 g, K_2HPO_4 -2.0 g, $MgSO_4.7H_2O$- 0.05 g, $CaCO_3$ - 0.02 g, $FeSO_4.7H_2O$ -0.01 g. Flasks were sterilized at 121 °C for 15 min and inoculated with two mycelia discs for 6 days at 120 rpm. Sampling was done at regular intervals for protease activity. The supernatant of culture filtrate was used as crude enzyme solution for protease assay.[20]

2.4.2 Protease assay

Protease activity was determined by Keay and Wildi method (1970).[21] The enzyme was added to 500 ml of 1% casein solution (pH of 7) followed by addition of 300 ml of phosphate buffer and incubated for 10 min at 37 °C. Addition of 1 ml of 10% (w/v) Trichloroacetic acid terminated the reaction and incubated at 32 °C for 10 mins. Subsequently 0.5 ml supernatant was mixed with 5 ml of 0.5 M

Na_2CO_3 and 1 ml of Folin-Ciocalteu's Phenol reagent: water (1:3 v/v) and incubated at room temperature for 30 mins. The optical density of the solution was determined at 660 nm and compared against the tyrosine standard. Protein concentration in the supernatant was studied by Lowry *et al.,* method (1951).[22]

3. Primary Optimization Stage: One Factor-At-A-Time Method

Statistical approaches offer ideal ways for process optimization studies in biotechnology.[23] A two-step statistical design of experiments was conducted for optimizing protease production by *Streptomyces* sp. LCJ1A. The first step involved screening variables for selecting the independent variables which influenced the output of protease production and the second step involved optimizing independent variables to test their interaction.

3.1. Optimization of protease production by Streptomyces sp. LCJ1A under submerged fermentation

Formulation and optimization of an appropriate culture medium is necessary.[24] In liquid culture medium, the effect of various factors on enzyme production was studied in different flasks, assessing one variable at a time, keeping all other factors constant. Once the optimization was carried out with respect to a factor, it was introduced into the experiment to optimize the next factor. The influence of various carbon sources like glucose, sucrose, maltose, fructose, mannitol, glycerol and lactose and its concentration, organic nitrogen source like peptone, yeast extract, urea and inorganic nitrogen sources like Sodium nitrate, Ammonium sulphate and Ammonium Nitrate, (0.5 to 5.0 g/L), natural inducers like groundnut oil cake, sesame oil cake, red gram husk, black gram husk, green gram husk, and chemical inducers like BSA, Gelatine and SDS on protease production were studied. High protease activity meant that the organism could produce a high amount of protease in that specific factor.[25]

3.1.1. Effect of carbon sources and its concentration on protease production

The influence of various carbon sources on protease production was studied by substituting starch in starch casein broth with different carbon sources (5 g/L). Fructose, glucose (Monosaccharides), maltose, sucrose, lactose (disaccharides), glycerol and mannitol (polysaccharides) were used. Flasks containing starch were maintained as control. After screening for the maximum protease producing carbon source, its concentration (0.5 g/L to 2.5 g/L) was optimized.

3.1.2. Effect of different nitrogen sources and its concentration on protease production

The effect of nitrogen source on protease production was studied by using them individually or as a mixture. Organic nitrogen sources such as urea, peptone and yeast extract, and inorganic sources like sodium nitrate, ammonium sulphate and ammonium nitrate were studied by using them separately. Flasks containing only casein were maintained as control. All the nitrogen sources were taken at 5 g/L of the basal media. After screening for the maximum protease producing nitrogen source, its concentration ranging from 0.5 to 2.5 g/L was optimized.

3.1.3. Effect of natural inducers and its concentration on protease production

The influence of natural inducers such as groundnut oil cake, sesame oil cake, green gram husk, black gram husk and red gram husk on protease activity was studied. Subsequently 0.5 to 3 g/L of natural inducers concentration was also studied.[26]

3.1.4. Effect of chemical inducers and its concentration on protease production

The influence of chemical inducers on protease production was studied. Different chemical inducers using such as BSA, Gelatine and SDS were amended in production medium at 0.5 to 3 g/L concentration.[27]

3.1.5. Effect of pH on protease production

The influence of pH on protease production by *Streptomyces* sp. LCJ1A was evaluated by adjusting medium pH from 4 to 10 using 0.1 N HCL and 0.1 N NaOH. The protease activity was calculated on alternate days.[28]

3.1.6. Effect of temperature on protease production

The influence of temperature on protease production by *Streptomyces* sp. LCJ1A was evaluated by adjusting temperature from 30 °C, 32 °C, 34 °C, 36 °C, 38 °C and 40 °C. The protease activity was determined on alternate days.[29]

3.2. Secondary Optimization Using Statistical Methods

For protease production, the statistical optimization of medium components was done in two stages:

3.2.1. Screening of important medium components by Plackett-Burman design

Depending on the results from the preliminary studies, a Plackett–Burman design was considered to study the medium components which influenced protease activity in *Streptomyces* sp. LCJ1A. It screens the components of the medium concerning their key function and not their interaction.[30] Total of 6 components were chosen with each variable being represented at two levels: -1 for low and +1 for high levels. Every factor was analysed at two separate concentration levels representing high and low set points. The selected factors for the study were maltose as carbon source, peptone as nitrogen source, pH, temperature, BSA and Green gram husk (Table 1).

Table 1: Medium components for protease production by Streptomyces sp. LCJ1A using Plackett-Burman Design (PBD)

Variables	Low level (-)	High level (+)
Maltose (g)	0.8	0.3
Peptone (g)	0.4	0.8
pH	7	0.5

Temperature	37	0.6
Green gram husk	0.3	0.6
BSA	1	0.4

The values are Mean ± S.D. of duplicates (n=12), (P<0.01) when compared with control

The experimental data was fitted according to Eq. (1) as a regression equation including individual and cross effect of each variable. From the Pareto- chart, the factors which showed highest positive effect on each category were assumed to have more influence on protease production and hence chosen for central composite design optimization of RSM.[31] Plackett-Burman Design follows the first-order polynomial model (Eq. 1) and is a two-level fractional design.

$$Y = \beta_0 + \sum \beta_n X_n \quad (1)$$

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.

3.2.2. Central Composite Design (CCD)

Response surface methodology helps in optimizing the significant components for enhancing protease production using CCD.[32] The influence of every variable on enzyme production was determined by 5 different levels and was implemented in 20 experiments where the influence of individual variables on production of protease was considered as response. The experiment design with the actual and coded values of the 4 variables were taken on various levels. The coded variables were maltose (A), peptone (B), pH (C) and temperature (D). These values were converted into their actual values to test the optimum range of factors for protease production. The experimental and predicted values for protease production by CCD were recorded. The relationship of the independent variables (A, B, C, D) and their response to protease production was subjected to Analysis of Variance (ANOVA). The results were calculated by the second order polynomial equation. The behaviour of the system was described by the following quadratic equation.

$$Y= \beta_0 +\beta_1 X_1 +\beta_2 X_2 +\beta_3 X_3 +\beta_4 X_4+\beta_{11} X_1^2 +\beta_{22} X_2^2 +\beta_{33} X_3+\beta_{44} X_4^2+\beta_{12} X_1 X_2 +\beta_{13} X_1 X_3+\beta_{14} X_1 X_4 +\beta_{23} X_2 X_3 +\beta_{24} X_2 X_4 +\beta_{34} X_3 X_4. \quad (2)$$

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.[33] Design Expert software is useful for multiple regression analysis and to construct the plots of the obtained data.[34] The fitted second order polynomial equation was also expressed as three dimensional surface plots to show the relationship of the experimental and response values. For validating the statistical model, the coefficients were determined by regression analysis and their level of significance. Protease production was subjected to analysis of variance (ANOVA) with a statistical software system (version 10, Stat-Ease) suitable for the experiment's design. Response surface plots were acquired to study the optimum levels of parameters for enhanced protease production.

3.2.3. Comparative analysis of original and optimized medium for the production of protease

The comparison was made between the original and optimized medium to demonstrate the efficiency of the optimized medium in protease production by *Streptomyces* sp. LCJ1A. The mycelial discs of the isolate were inoculated in a separate series of experiments in 100 mL of original and modified medium and incubated for 2-12 days. The protease activity was then assayed in alternate days and was measured by reading absorbance at 660 nm in UV- Spectrophotometer (ELICO SL159) (Mention the name of Model and Make).

4. STATISTICAL ANALYSIS

Each experiment was carried out in duplicates. The mean and standard deviation (SD) were calculated and data was expressed as Mean ± SD of the duplicates. The statistical analysis for each experiment was carried out using one way ANOVA followed by Tukey's multiple comparison tests with statistical significance is set at $p < 0.01$. All the analysis was performed with SPSS 11.5. Design Expert 8.0.7.1 (State-Ease, Inc) was used for the experimental designs and subsequent regression analysis of the experimental data.

5. RESULTS & DISCUSSION

5.1 Screening and Identification of isolates for protease Production

Fifteen actinomycetes isolates were collected and cultured on SCA plates and examined for protease activity. Among the 15 isolates, *Streptomyces* sp. LCJ1A was further selected for quantitative studies as the isolate produced maximum clear proteolytic zone. In the qualitative assay, the isolate showed large brown coloured colonies on the SCA medium (figure 1) suggesting the protease activity and displayed the largest region suggesting its higher protease development capacity relative to other isolates. This is in accord with study by A.H. Mohamedin, about qualitative screening results of protease enzyme production by *Streptomyces* strain.[35] Further, the Quantitative assay results showed that the isolate *Streptomyces* sp. LCJ1A exhibited higher protease activity 70.59 IU/mL in Starch casein broth. The LCJ1A isolate was selected for further optimization of its higher protease activity in liquid cultures using CCD and RSM. In a similar study by Sathya, 205 actinomycete samples were isolated from Muthupet mangroves and most of them were found to be *Streptomyces* sp.[36]

Fig 1: Colony Morphology of Streptomyces sp. LCJ1A

5.2 Molecular characterization by 16S rRNA sequencing

Genomic analysis of *Streptomyces* sp. LCJ1A was done. The universal primer sequences 16 S-27 F and 16 S-1492 R were used for DNA sequencing by Sanger's dideoxy method using big dye terminator v3.1. The isolate sequences was compared with those in the nucleotide sequence database of the National Center for Biotechnology Information (NCBI), using the Basic Local Alignment Search Tool (BLAST). The sequence was submitted in NCBI using the 16S rRNA submission method via BankIt and obtained the accession number. The GENBANK accession number for *Streptomyces* sp. LCJ1A is KU 870428. Phylogeny was constructed using MEGA 6.0. software (Figure 2) and showed a 99% homology with *Streptomyces exfoliatus*, when matched with genetically similar sequences in a NCBI database. In a similar study by MS Palla, novel actinomycete strain isolated from Mangrove soils and based on 16S rRNA sequencing the strain was identified as *Streptomyces hydrogenans*.[37]

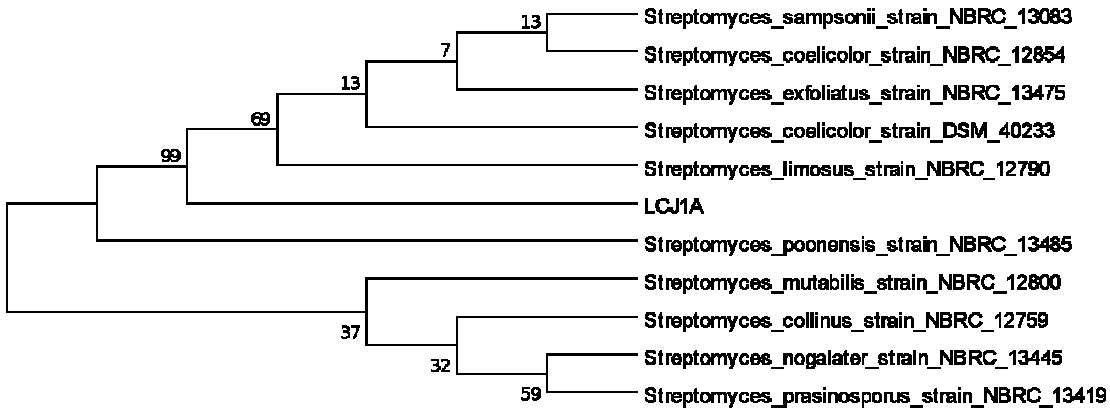


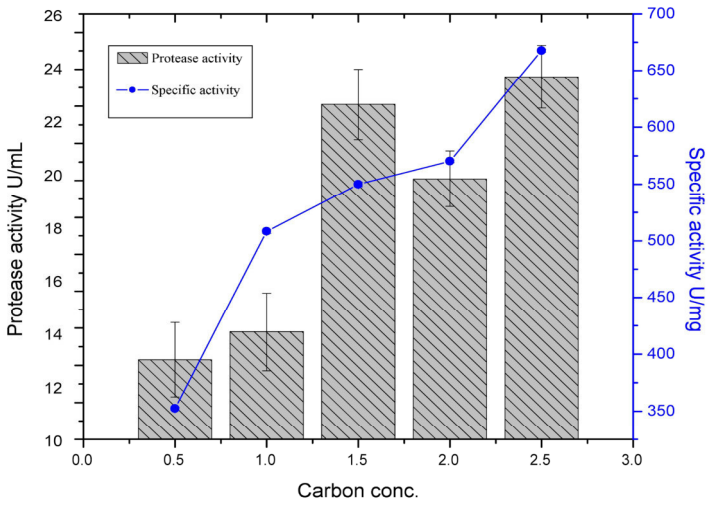
Fig 2: Neighbor-Joining tree of the ITS region of strains LCJ1A and with higher similarity from the genbank selected from the results of a BLASTn search

5.3 Selection of liquid medium for protease production

Submerged fermentation allows the organisms to grow in a liquid medium. Submerged fermentation (SmF) involving the cultivation of microbes in liquid media has several benefits, like efficiency in regulating various criteria and scaling up procedures.[38] Selection of a suitable liquid medium for *Streptomyces* sp. LCJ1A protease production was studied using six different media PPB, GB, MYEB, SCB, MNGA and GYPB. Protease activity was observed in all the different media. Among the six media tested, SCB medium supported enhanced protease production, therefore SCB was selected as the suitable medium for protease production. The effect of various carbon and nitrogen sources can be studied by substituting the corresponding component in the production medium.[39]

5.3.1 Effect of different carbon sources and their concentration on protease production

Optimizing the carbon source and its concentration is a critical factor in the regulation of protease production. The influence of various carbon sources on protease production was studied by replacing starch with suitable alternatives in the production medium. Each carbon source studied, induced protease production. Maltose showed the best protease yield of 18.93 U/mL enzyme activity. The study on the influence of different concentrations of maltose revealed that 2.5 g/L of maltose was the best concentration with 23.91 U/mL protease activity (Figure 3), and addition after this resulted in reduced protease production. Similar research conducted by RS Prakasham, showed that maltose favoured protease production from *Bacillus circulans*.[40]



The values are Mean ± S.D. of duplicates (P<0.01)

Fig 3: Optimization of concentration of carbon source for Streptomyces sp. LCJ1A in Starch casein broth on peak day

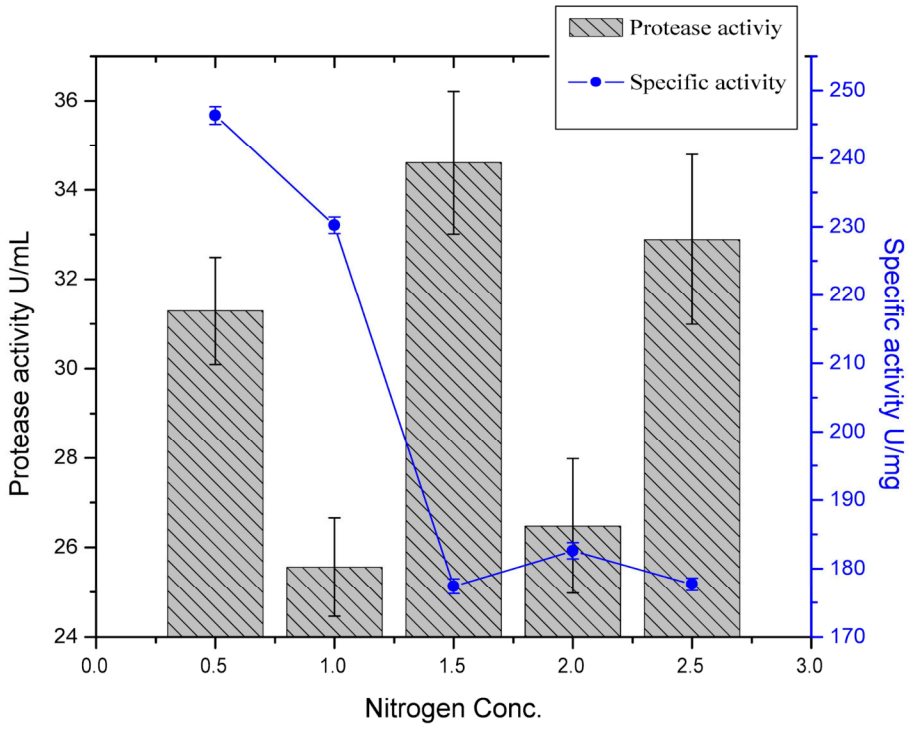
5.3.2 Effect of different nitrogen sources and their concentration on protease production

Enzyme production by microbes is influenced by both nature and concentration of nitrogen sources. Hence they are considered as important factors for regulation of enzyme production. In most cases, nitrogen source used in the medium affects the growth as well as protease production.[41] Casein was the actual nitrogen source in the production medium. Among the tested nitrogen sources, peptone showed 32.55 U/mL protease activity (Table 2). The inorganic nitrogen sources were not effective as the organic nitrogen sources. The study on the different concentrations of peptone (0.5 to 2.5 g/L) on protease production showed that 34.61 U/mL of highest enzyme activity was seen at 1.5 g/L peptone (Figure 4). In a similar study by Parthasarathy, Sodium nitrate was found to enhance protease production by *Streptomyces* sp. LCJ12A.[42]

Table 2: Effect of different carbon sources, nitrogen sources, natural and chemical inducers in the culture medium for protease production by Streptomyces sp. LCJ1A

Nutrient Source	Protease yield
Carbon source (5 g/L)	
Sucrose	15.91±1.5
Mannitol	16.71±1.2
Glucose	16.73±1.3
Lactose	18.75±1.2
Glycerol Fructose	16.05±1.5 17.73±1.3
Maltose	18.93±1.4
Nitrogen source (3 g/L)	
Urea	31.87±1.6
peptone	32.55±1.3
Ammonium nitrate	24.87±1.2
Yeast extract	26.36±1.2
Ammonium sulphate	25.79±1.5
Sodium nitrate	24.12±1.7
Natural Inducer (3 g/L)	
Green Gram husk	31.40±1.1
Red Gram husk	23.61±1.1
Black Gram husk	28.19±1.1
Sesame oil cake	24.99±1.0
Groundnut oilcake	21.32±1.2
Chemical Inducer (3 g/L)	
BSA	23.72±1.3
Gelatine	20.40±1.2
SDS	11.69±1.0

± indicates the standard deviation of duplicates (n=21), (P<0.01) when compared with control

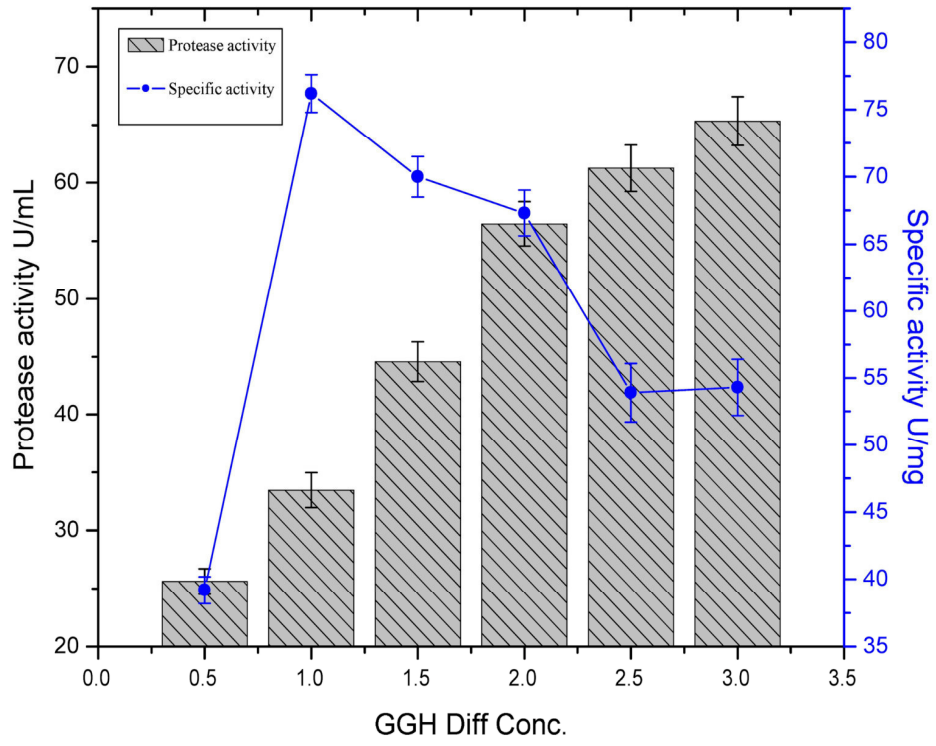


The values are Mean ± S.D. of duplicates (P<0.01)

Fig 4: Optimization of concentration of nitrogen source for Streptomyces sp. LCJ1A in Starch casein broth on peak day

5.3.3 Effect of different natural inducers and their concentration on protease production

The choice of an appropriate inducing substrate is very much needed for protease production.[43] Addition of the natural inducer, green gram husk to the medium favoured protease activity of 31.40 U/mL. Further, when different concentrations of green gram husk (0.5 to 3.0 g/L) was tested, *Streptomyces* sp. LCJ1A showed enhanced protease activity of 65.72 U/mL at 3.0 g/L of GGH (Figure 5). In a similar study by Srinivasan *et al.,* 2% casein was found to be a good inducer for protease production by *Conidiobolus* sp.[44]

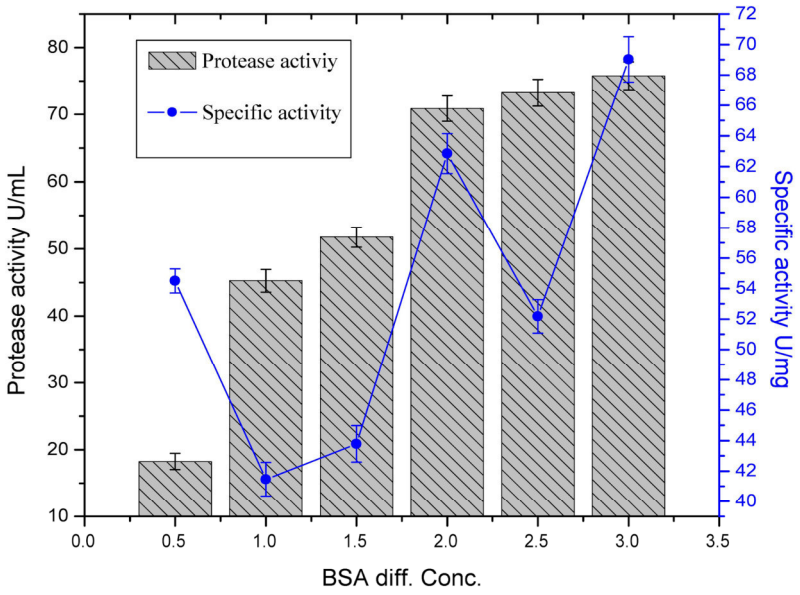


The values are Mean ± S.D. of duplicates (P<0.01)

Fig 5: Optimization of concentration of natural inducer for Streptomyces sp. LCJ1A in Starch casein broth on peak day

5.3.4 Effect of different chemical inducers and their concentration on protease production

The influence of chemical inducers on protease production was studied by supplementing BSA, Gelatine and SDS to the production medium. BSA supported the maximum enzyme activity of 23.72 U/mL protease. Gelatine and SDS induced protease activity to a certain extent. Concentrations over a test range of 0.5 to 3.0 g /L were studied. 3.0 g/L of BSA showed significant 75.58 U/mL protease activity (Figure 6). In one of the studies by Ferid Abidi, marine *Spirulina algae* was used as an inducer for enhanced protease production from *Botrytis cinereal*.[45]



The values are Mean ± S.D. of duplicates (P<0.01)

Fig 6: Optimization of concentration of chemical inducers for Streptomyces sp. LCJ1A in Starch casein broth on peak day

5.3.5 Effect of different pH on protease production

To study the influence of pH on protease production, pH was adjusted from 4 to 10 using 0.1N HCL and 0.1N NaOH. At pH 10, 42.31 U/mL of maximum protease activity was recorded. The physical parameters like pH and temperature have been found to play a significant role in enzyme production. The study proved that medium with a pH 10 favoured the protease production in SCB and the protease enzyme was found to be alkaline in nature. This is in accord with the study by Pradeep Palsaniya about protease production by bacteria.[46]

5.3.6 Effect of different temperature on protease production

Different ranges of temperature from 30°C, 32°C, 34°C, 36°C, 38°C and 40°C were studied to know the influence of temperature on protease production. Maximum protease activity of 150.81 U/mL was noted at 34°C. Temperatures higher than 34°C showed decreased enzyme activity. Therefore, temperature conditions ranging from 32°C to 34°C were considered optimal for protease production. In a study conducted by R. Seeta Laxman, maximum protease activity by *Conidiobolus coronatus* was found at 28°C.[47]

5.4 Secondary Optimization Using Statistical Methods

5.4.1 Plackett-Burman Design

Statistical methods for medium optimization are useful and important in industrial biotechnological processes. Plackett-Burman design is a promising experimental design for the identification of significant factors affecting productivity.[48] The results of conventional method showed the noteworthy factors which controlled protease production and were screened by Plackett-Burman Design (PDB). The first statistical optimization step identified the important factors using a 14 run Plackett-Burman design. Variations from 16.74 U/mL to 52.34 U/mL in the 14 runs were observed, within the range of the 6 variables chosen for protease production (Table 3).

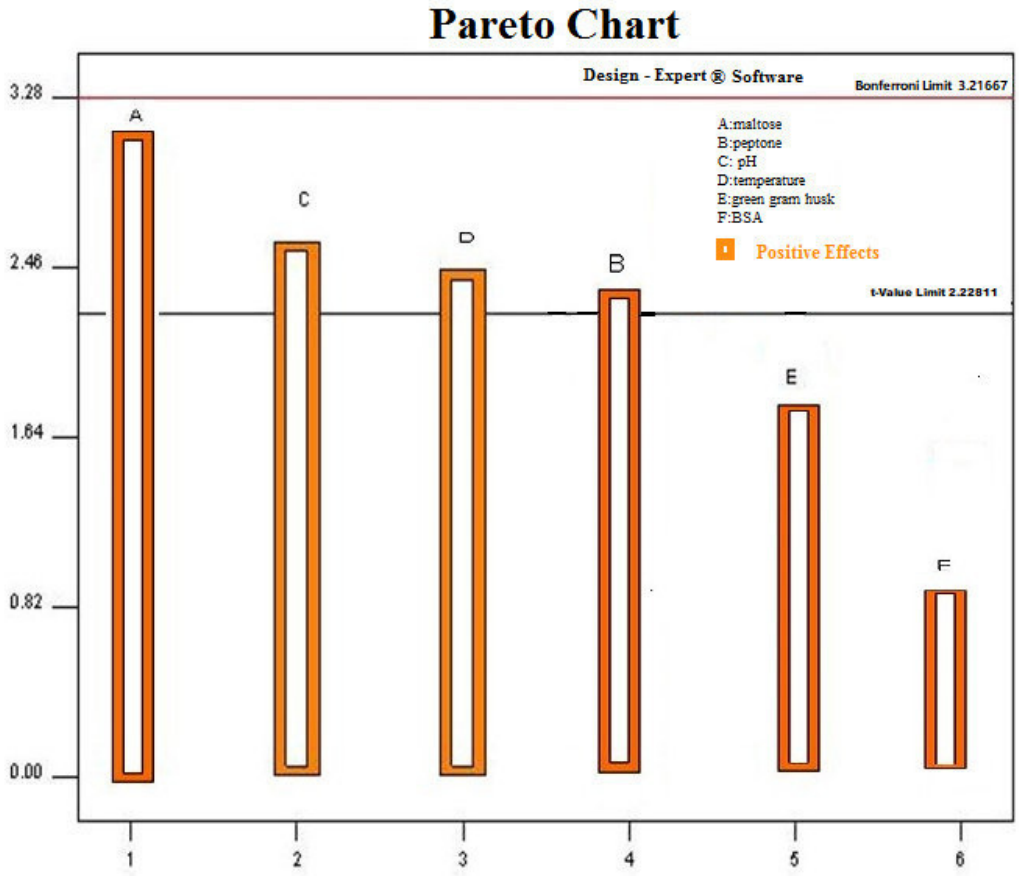
Table 3: Plackett- Burman Design matrix, their observed and predicted values for protease production by Streptomyces sp. LCJ1A

Run	Maltose (g)	Peptone (g)	pH	Temp	Green gram husk (g)	BSA (g)	protease activity (U/mL)	Run
							Observed value	Predicted value
1	1.5	1.0	6.5	30	1.0	2.0	43.12	41.47
2	1.0	1.0	6.0	34	2.5	2.5	29.40	23.64
3	1.5	0.5	11.0	36	0.5	2.5	32.13	31.09
4	0.5	1.0	4.5	24	2.0	1.5	49.19	36.32

5	2.0	2.5	5.0	30	3.0	1.5	40.96	32.44
6	2.5	2.0	4.5	32	1.5	1.0	32.11	32.04
7	0.5	3.0	3.5	26	2.0	1.0	38.74	29.88
8	1.0	1.5	8.0	28	1.5	2.5	41.62	40.11
9	1.5	1.0	8.5	32	0.5	0.5	52.34	44.56
10	2.0	2.5	5.5	38	2.0	0.5	23.11	23.72
11	0.5	2.5	4.0	36	2.5	1.0	32.76	27.97
12	2.5	0.5	7.0	38	2.5	0.5	23.18	19.28
13	2.5	3.0	10.0	40	0.5	2.5	16.74	27.85
14	3.0	1.5	10.5	36	2.5	2.5	20.17	22.45

The values are Mean ± S.D. of duplicates (P<0.01) ± indicates the standard deviation of duplicates (n=6), (P<0.01) when compared with control

The differences represented the importance of medium optimization to attain increased yield. The variables controlling the order of significance for protease production are shown in Pareto chart (Fig 7). Maltose showed highest significance followed by pH, temperature and peptone. Subsequently, these 4 factors were chosen to achieve maximum protease efficiency using optimization. The level of factors (Maltose, pH, temperature and peptone) and the impact of their interactions on enzyme production was studied by central composite design using RSM. Similarly a study by Duraikannu found that several nutritional and physiologic parameters influenced protease production by *Streptomyces radiopugnans*_VITSD8 strain.[49]



The values are Mean ± S.D. of duplicates (P<0.01)

Fig 7: Pareto chart showing the effect of six factors on protease production by Streptomyces sp. LCJ1A

5.4.2 Response surface methodology (RSM)

Based on the Plackett- Burman design, a central composite design was used to determine the combined effect of the four variables. Maltose, peptone, pH and temperature interactions were studied by CCD. In the CCD design 29 runs replicated at 5 central points were performed. The predicted and observed responses along with the design matrix are given in Table 4. The interactions between the 4 variables were determined by fitting a second order polynomial equation to data obtained from the 29 runs. The results were analysed by ANOVA.

Table 4: FCCD Results of Streptomyces sp. LCJ1A using 4 independent variables

RUN	Maltose	Peptone	pH	Temp	Observed Value	Predicted value
1	-1	0	0	-1	158.04	159.69
2	0	0	-1	0	92.49	100.22

3	0	0	0	0	42.98	44.07
4	1	0	0	1	136.89	139.82
5	0	0	-1	0	69.33	70.09
6	0	0	0	0	156.81	157.02
7	1	1	0	1	60.99	61.48
8	0	-1	-1	0	113.43	124.18
9	0	1	0	0	151.05	150.33
10	0	0	0	0	148.11	150.93
11	0	0	-0	0	124.55	145.59
12	0	1	-1	0	109.38	122.39
13	-1	-1	0	-1	57.91	58.94
14	0	0	0	0	56.59	57.06
15	0	-1	1	-1	147.14	45.11
16	0	1	1	1	51.92	52.52
17	-1	1	0	1	158.14	166.34
18	1	0	0	0	82.03	62.59
19	0	-1	0	-1	101.55	99.11
20	-1	0	0	0	62.15	61.93
21	-1	0	1	0	52.84	56.13
22	1	0	1	0	99.11	102.38
23	1	-1	0	-1	60.33	62.93
24	1	0	-1	0	94.93	105.46
25	0	0	1	0	186.92	190.67
26	0	0	1	0	62.19	63.11
27	-1	0	-1	0	151.98	152.67
28	0	-1	0	-1	150.45	163.59
29	0	1	0	1	101.58	101.58

± indicates the standard deviation of duplicates (n=6) and (P<0.01) when compared with control

The regression equation gave the levels of protease activity as the function of maltose, peptone, pH and temperature which are shown in terms of coded factors in the equation as:

$$Y = 167.25-7.13X_1-9.68X_2-5.65X_3-66.87X_1X_2-52.49\ X_1X_3+ 51.42\ X_2X_3- 23.96\ X_1X_4- 32.94\ X_1^2-23.86X_2^2-32.11\ X_3^2$$

Where Y is the protease activity (U/mL), A, B, C, D are Maltose, peptone, pH and temperature, respectively. This is in accord with the second order quadratic equation studied by Khurana.[50] The goodness of fit of the model was checked by the determination coefficient (R^2).[51] Fisher F-test had a very low probability value (P model >F = 0.0001). The coefficient of determination (R^2) was calculated to be 0.964. A value of $R^2 > 0.75$ indicates the aptness of the model. The value of the adjusted determination coefficient was Adjusted R^2 = 0.917. A relatively low coefficient variation, CV=1.19% was observed. The three dimensional (3D) graphs were drawn depending on the final model to study the favourable concentration for each factor and the interaction among the factors to maximize protease production (Figure. 8 and Figure 9). Each figure shows the influence of 2 variables on the protease production, while other variables were fixed at zero level. Protease production behaviour of *Streptomyces* sp. LCJ1A, interaction effect, main effect and squared effect (nonlinear) of 4 factors, at various concentrations were also shown in three-dimensional plot. Both maltose and peptone exhibited higher protease production. Hence it is clearly understood that the nature of carbon and nitrogen sources influence protease production. This is in accord with study conducted by LVA Reddy.[52] The interaction effects of maltose and peptone were found to be extremely important (P-value=0.01), suggesting that both components were crucial for protease production by *Streptomyces* sp. LCJ1A. The favourable concentrations of maltose and peptone were found to be 2.5 g/L and 1.5 g/L in the surface response plot. 190.67 U/mL of the maximum predicted enzyme can be produced by using optimal concentration of medium components. Several studies also reported similar improved protease production by employing RSM. [53]

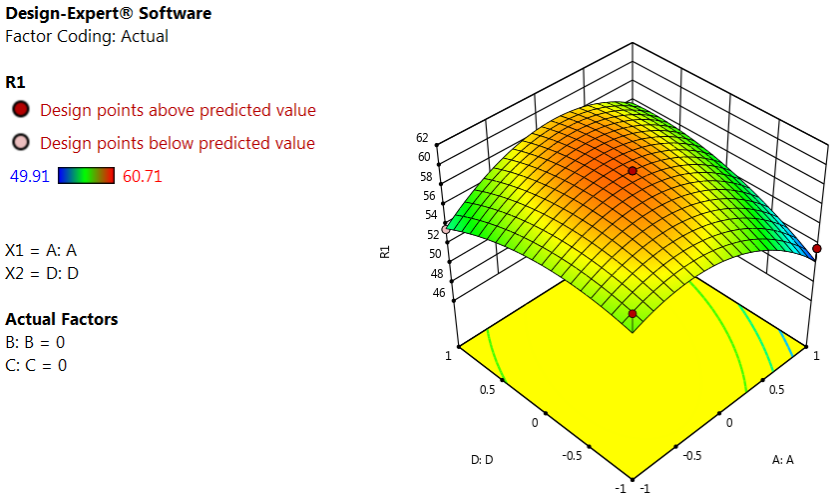


Fig 8: Three dimensional graph of the Interaction between maltose and temperature on the protease production by Streptomyces sp. LCJ1A

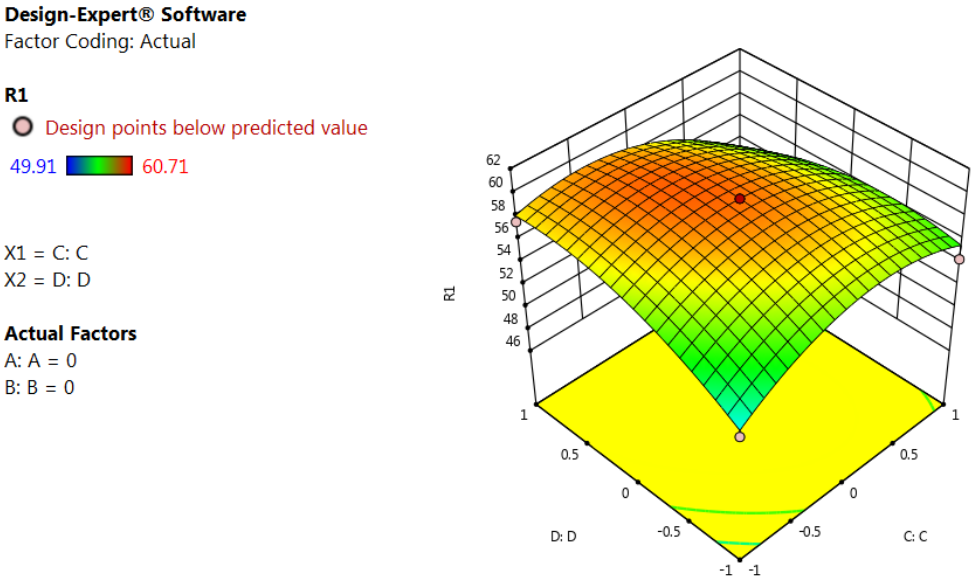


Fig 9: Three dimensional graph of the Interaction between peptone and pH on the protease production by Streptomyces sp. LCJ1A

5.4.3 Validation of the model

Validation of the optimized factors was studied under conditions predicted by the model. A protease production of 186.92 U/mL was obtained in the experimental study which was close to the predicted value of 190.67 U/mL, thereby validating the model. The optimization of medium components ensures a balance among the different medium components and hence reduces the amount of unused components.[54] The conventional methods of optimization involves altering one factor besides fixing the others. This method is expensive, time consuming and it is not clear what interactions are among the factors. *Streptomyces* sp. nutritional requirements have a major role during the metabolite synthesis process.[55] It is well documented that the extracellular enzyme production by microbes is strongly controlled by media components.[56] Among the different nutritional requirements, the carbon sources and the nitrogen sources are usually considered to be essential metabolic factors, and several examples of the production of metabolites in media with optimized content of these components are also explained in the literature.[57,58] The major factors in "one factor at a time" are carbon, nitrogen, inducers, pH and temperature. Maltose induced favourable protease activity in this study. The presence of more sugar in the medium has been studied by Patel for enhanced protease production.[59] The high production rate of secondary metabolites explains the increased protease activity in media containing these simple sugars. Proteases show different responses to carbon sources and their concentration in the nutrient medium. Peptone, an organic nitrogen source favoured increased protease activity in *Streptomyces* sp. LCJ1A. This is similar to the study conducted by Pant G, showed that peptone enhanced protease production from *Bacillus subtilis*.[60] Many actinomycete strains that generate enzymes of great industrial potential were isolated from diverse extreme environments.[61] In *Streptomyces galbus*, organic nitrogen production increased enzyme activity as reported by Sivakumar *et al*., suggesting that certain essential amino acids could not be synthesized when inorganic nitrogen sources are used.[62] Greater amounts of protease can be obtained under high nitrogen concentration. Previous studies by Genckal *et al.,* described that the type of nitrogen and its concentrations strongly influenced enzyme production and cell growth. [63] An effective approach for enhanced protease activity is the supplementation of the

nutrient medium with suitable inducers. In a similar study by Singh and Bajaj, when soybean cake was used as inducer in *Bacillus licheniformis,* enzyme activity increased.[64] RSM studies on protease production by Benluvankar, showed that certain chemical inducers like Casein can be used for maximizing enzyme production.[65] Organic nutrients provide required vitamins, nutrients and minerals for enhanced protease production by different microorganisms.[66] In a study by Kuppusamy and kumar, traditional one variable at a time method was used to find the important nutritional factors that enhanced enzyme production.[67] RSM studies by JR Dutta showed that the combined effect of pH and temperature may not change from single variable optimized conditions.[68] RSM is an effective method for optimizing the media components. The protease production using the 'one factor at a time' approach shows the interactions and effects of various factors on enzyme production. PBD was used for studying the most important fermentation parameters which enhanced enzyme activity.[69] It is used to find out the important variables controlling protease production. Based on PBD, the factors A (Fructose), B (peptone) and D (temperature) were found to influence protease production. The four most significant media components maltose, peptone, pH and temperature were further optimized using CCD. The influence of media components on protease production was analysed using ANOVA. The determination coefficient (R^2) value of 0.964 implies that 96.4% of experimental data was compatible with the data predicted by the model. The value of the adjusted determination coefficient (AdjR^2 = 0.917) demonstrates a high significance of the model. It is very well known that R^2 value is always between 0 and 1. And if R^2 value is close to 1.0, then it can be concluded that the model is strong and predicts the response in a better way.[70] A relatively low coefficient variation (CV=1.19%) confirmed the reliability of the experiment performed. The ANOVA results revealed that these 4 factors influenced enzyme production and the regression model showed that the model is very significant. The validation study proved that the values predicted by the model were in good agreement with the results obtained. With the optimization of the culture components, a 10 fold increase in enzyme production was achieved.

6. CONCLUSION

In this study, Plackett-Burman and Central composite Design were used for optimizing culture media for enhanced protease production by using *Streptomyces* sp. LCJ1A. Medium components such as maltose, peptone and green ram husk influenced the protease production significantly. These factors were chosen for further optimization studies using RSM. The optimized medium is helpful for further large scale production of protease using *Streptomyces* sp. LCJ1A. Thus results of this study indicate that optimization by sequential optimization method using both conventional and statistical methods are an effective way of optimizing and maximizing protease production even up to 10 folds. Therefore, optimization of protease production by conventional methods for *Streptomyces* sp. LCJ1A proved that the isolate showed a good enzyme activity. Optimization of culture conditions and essential nutrients for protease production under submerged fermentation will help in the large scale production of the enzyme and the organism can further be used for studying cost effectiveness using agro-industrial substrate besides purification and characterization studies.

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8. AUTHORS CONTRIBUTION STATEMENT

Ms. Swarna Dhavala worked as Junior Research Fellow in the project and Dr. J. Joel Gnanadoss was the Principal Investigator. The concept was developed, guided and supervised by Dr. J. Joel Gnanadoss.

9. CONFLICT OF INTEREST

Conflict of interest declared none.

10. REFERENCES

1. Hamza TA. Bacterial protease enzyme: Safe and good alternative for industrial and commercial use. Int. J. Chem. Biomol. Sci. 2017;3(1):1-0. Available from: https://pdfs.semanticscholar.org/7943/ad6326fc6577937dcd4dc74a3c2601b3c70a.pdf
2. 2. Jenifer JA, Remya R, Velmurugan S, Michaelbabu M, Citarasu T. Streptomyces castaneoglobisporus AJ9, A haloalkaliphilic actinomycetes isolated from solar salt works in southern India and its pharmacological properties. Indian J Geomarine Sci. 2018 Feb; 47(02): 475-488 Available from: http://nopr.niscair.res.in/handle/123456789/43569
3. Latha S, Sivaranjani G, Dhanasekaran D. Response surface methodology: A non-conventional statistical tool to maximize the throughput of Streptomyces species biomass and their bioactive metabolites. Crit Rev Microbiol. Sep 2017;43(5):567-82. doi: 10.1080/1040841X.2016.1271308, PMID 28129718.
4. Sumantha A, Deepa P, Sandhya C, Szakacs G, Soccol CR, Pandey A. Rice bran as a substrate for proteolytic enzyme production. Braz Arch Biol Technol. 2006 Sep;49(5):843-51. doi: 10.1590/S1516-89132006000600019.
5. Singh J, Batra N, Sobti RC. Serine alkaline protease from a newly isolated Bacillus sp. SSR1. Process Biochem. 2001 Mar 1;36(8-9):781-5. doi: 10.1016/S0032-9592(00)00275-2.
6. Beg QK, Gupta R. Purification and characterization of an oxidation-stable, thiol-dependent serine alkaline protease from Bacillus mojavensis. Enzyme Microb Technol. 2003 Feb 3;32(2):294-304. doi: 10.1016/S0141-0229(02)00293-4.
7. Chu WH. Optimization of extracellular alkaline protease production from species of Bacillus. J Ind Microbiol Biotechnol. 2007 Mar 1;34(3):241-5. doi: 10.1007/s10295-006-0192-2, PMID 17171551.
8. Oskouie SFG, Tabandeh F, Yakhchali B, Eftekhar F. Response surface optimization of medium composition for alkaline protease production by Bacillus clausii. Biochem Eng J. 2008 Apr 1;39(1):37-42. doi: 10.1016/j.bej.2007.08.016.

9. Sinha S, Sinha S. Studies on the production of acid protease by submerged fermentation. Int J Food Eng. 2009 Mar 16;5(1). doi: 10.2202/1556-3758.1338.
10. Gao H, Liu M, Liu J, Dai H, Zhou X, Liu X, Zhuo Y, Zhang W, Zhang L. Medium optimization for the production of avermectin B1a by Streptomyces avermitilis 14-12A using response surface methodology. Bioresour Technol. 2009 Sep 1;100(17):4012-6. doi: 10.1016/j.biortech.2009.03.013, PMID 19356927.
11. Unni S, Prabhu AA, Pandey R, Hande R, Veeranki VD. Artificial neural network-genetic algorithm (ANN-GA) based medium optimization for the production of human interferon gamma (hIFN-γ) in Kluyveromyces lactis cell factory. Can J Chem Eng. Apr 2019;97(4):843-58. doi: 10.1002/cjce.23350.
12. Vidya B, Gomathi D, Kalaiselvi M, Ravikumar G, Uma C. Production and optimization of amylase from Penicillium chrysogenum under submerged fermentation. World J Pharm Res. 2012 Jul 10;1(4):1116-25.
13. Singh SK, Singh SK, Tripathi VR, Khare SK, Garg SK. Comparative one-factor-at-a-time, response surface (statistical) and bench-scale bioreactor level optimization of thermoalkaline protease production from a psychrotrophic Pseudomonas putida SKG-1 isolate. Microb Cell Fact. 2011 Dec 1;10(1):114. doi: 10.1186/1475-2859-10-114, PMID 22204659.
14. Rao RS, Prakasham RS, Prasad KK, Rajesham SS, Sarma PN, Rao LV. Xylitol production by Candida sp.: parameter optimization using Taguchi approach. Process Biochem. 2004 Apr 30;39(8):951-6. doi: 10.1016/S0032-9592(03)00207-3.
15. Kar S, Ray RC. Statistical optimization of alpha-amylase production by Streptomyces erumpens MTCC 7317 cells in calcium alginate beads using response surface methodology. Pol J Microbiol. 2008;57(1):49-57. PMID 18610656.
16. Sarkar G, Suthindhiran K. Extraction and characterization of alkaline protease from Streptomyces sp. GS-1 and its application as dehairing agent. Biocatal Agric Biotechnol. 2020 Mar 28:. doi: 10.1016/j.bcab.2020.101590.
17. Ramesh S, Mathivanan N. Screening of marine actinomycetes isolated from the Bay of Bengal, India for antimicrobial activity and industrial enzymes. World J Microbiol Biotechnol. 2009 Dec 1;25(12):2103-11. Available from: https://link.springer.com/article/10.1007/s11274-009-0113-4
18. Hamed MM, Abdelfattah LS, Fahmy NM. Antimicrobial Activity of Marine Actinomycetes and the Optimization of Culture Conditions for the Production of antimicrobial Agent (s). J Pure Appl Microbiol. 2019;13(4):2177-88. doi: 10.22207/JPAM.13.4.30.
19. Joo HS, Chang CS. Production of an oxidant and SDS-stable alkaline protease from an alkaophilic Bacillus clausii I-52 by submerged fermentation: feasibility as a laundry detergent additive. Enzyme Microb Technol. 2006 Jan 3;38(1-2):176-83. doi: 10.1016/j.enzmictec.2005.05.008.
20. Balachandran C, Duraipandiyan V, Ignacimuthu S. Purification and characterization of protease enzyme from actinomycetes and its cytotoxic effect on cancer cell line (A549). Asian Pac J Trop Biomed. 2012 Feb 1;2(2):S1138-46. doi: 10.1016/S2221-1691(12)60374-8.
21. Keay L, Wildi BS. Proteases of the genus Bacillus. I. Neutral proteases. Biotechnol Bioeng. 1970 Mar;12(2):179-212. doi: 10.1002/bit.260120205, PMID 5469582.
22. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. J Biol Chem. 1951 Nov;193(1):265-75. PMID 14907713.
23. Gupta R, Beg QK, Lorenz P. Bacterial alkaline proteases: molecular approaches and industrial applications. Appl Microbiol Biotechnol. 2002 Jun 1;59(1):15-32. doi: 10.1007/s00253-002-0975-y, PMID 12073127.
24. Braga AR, Gomes PA, Kalil SJ. Formulation of culture medium with agro-industrial waste for β-galactosidase production from Kluyveromyces marxianus ATCC 16045. Food and Bioproc Tech. 2012 Jul 1;5(5):1653-63. doi:10.1007/s11947-011-0511-0
25. Razzaq A, Shamsi S, Ali A, Ali Q, Sajjad M, Malik A, Ashraf M. Microbial proteases applications. Front Bioeng Biotechnol. Jun 2019;7(7):110. doi: 10.3389/fbioe.2019.00110, PMID 31263696.
26. Jenitta XJ, Priya SE, Gnanadoss JJ. Optimization of Culture Conditions and Inducers for Improved Protease Production by Penicillium griseofulvum LCJ231 under Submerged Fermentation. Int J Adv Biotechnol Res. Jan 2015 1;6(2):152-60. Available from: http://www.bipublication.com
27. Chandrasekaran M, Chandrasekar R, Chun SC, Sathiyabama M. Isolation, characterization and molecular three-dimensional structural predictions of metalloprotease from a phytopathogenic fungus, Alternaria solani (Ell. and Mart.) Sor. J Biosci Bioeng. 2016 Aug 1;122(2):131-9. doi: 10.1016/j.jbiosc.2015.12.021, PMID 26924427.
28. Sunil LH, Manish SB, Neela MB. Isolation, purification and partial characterization of thermostable serine alkaline protease from a newly isolated Bacillus thuringinsis-SH-II-1A. Afr J Biotechnol. 2018 Feb 14;17(7):178-88. doi: 10.5897/AJB2015.14831.
29. Abdullah Al-Dhabi N, Ali Esmail G, Mohammed Ghilan A, Valan Arasu M, Duraipandiyan V, Ponmurugan K. Characterization and fermentation optimization of novel thermo stable alkaline protease from Streptomyces sp. Al-Dhabi-82 from the Saudi Arabian environment for eco-friendly and industrial applications. J King Saud Univ Sci. 2020;32(1):1258-64. doi: 10.1016/j.jksus.2019.11.011.
30. Plackett RL, Burman JP. The design of optimum multifactorial experiments. Biometrika. 1946 Jun 1;33(4):305-25. doi: 10.1093/biomet/33.4.305.
31. Abdel Wahab WA, Ahmed SA. Response surface methodology for production, characterization and application of solvent, salt and alkali-tolerant alkaline protease from isolated fungal strain Aspergillus niger WA 2017. Int J Biol Macromol. 2018 Aug 1;115:447-58. doi: 10.1016/j.ijbiomac.2018.04.041, PMID 29678788.
32. Khan S, Misra AK, Tripathi CK, Mishra BN, Bihari V. Response surface optimization of effective medium

constituents for the production of alkaline protease from a newly isolated strain of Pseudomonas aeruginosa. Ind Exp Biol. 2006 Feb; 44:151-156. Available from: http://nopr.niscair.res.in/handle/123456789/6369

33. Li N, Jiang Y, Li M, Luo X, Liu Y, Kan H, Zhang J, Zhao P. Optimization of Depolymerization Process of Polymeric proanthocyanidins from the Barks of Pinus kesiya var. langbianensis. Sci Silvae Sin. 2017 Feb 25;53(2):110-6. doi: 10.11707/j.1001-7488.201702130.
34. Reddy LV, Wee YJ, Yun JS, Ryu HW. Optimization of alkaline protease production by batch culture of Bacillus sp. RKY3 through Plackett–Burman and response surface methodological approaches. Bioresour Technol. 2008 May 1;99(7):2242-9. doi: 10.1016/j.biortech.2007.05.006, PMID 17596938.
35. Mohamedin AH. Isolation, identification and some cultural conditions of a protease-producing thermophilic Streptomyces strain grown on chicken feather as a substrate. Int Biodeterior Biodegrad. 1999 Mar 1;43(1-2):13-21. doi: 10.1016/S0964-8305(98)00061-4.
36. Sathya R, Ushadevi T. Industrially important enzymes producing Streptomyces species from mangrove sediments. Int J Pharm Pharm Sci. 2014;6(10):233-7.
37. Palla MS, Guntuku GS, Muthyala MKK, Pingali S, Sahu PK. Isolation and molecular characterization of antifungal metabolite producing actinomycete from mangrove soil. Beni Suef Univ J Basic Appl Sci. 2018 Jun 1;7(2):250-6. doi: 10.1016/j.bjbas.2018.02.006.
38. Ho HL, Abduljubar MH. Strain development of Aspergillus brasiliensis using physical and chemicals mutagenesis for possible overproduction of xylanase, amylase, protease and cellulase under submerged fermentation (SmF). Microbiol Res J Int. 2016 Jan 21;12(5):1-19. doi: 10.9734/BMRJ/2016/22953.
39. Chellapandi P. Production and preliminary characterization of alkaline protease from Aspergillus flavus and Aspergillus terreus. J Chem. 2010 Apr 1;7(2):479-82. doi: 10.1155/2010/502583.
40. Prakasham RS, Subba Rao Ch, Sreenivas Rao R, Sarma PN. Alkaline protease production by an isolated Bacillus circulans under solid-state fermentation using agroindustrial waste:process parameters optimization. Biotechnol Prog. 2005;21(5):1380-8. doi: 10.1021/bp050095e, PMID 16209541.
41. Bazarzhapov BB, Lavrent'ev EV, Dunaevskiĭ IaE, Bilanenko EN, Namsaraev BB. Extracellular proteolytic eyzymes of microscopic fungi from thermal springs of the Barguzin Valley (Northern Baikal region). Prikl Biokhim Mikrobiol. 2006;42(2):209-12. doi: 10.1134/S000368380602013X. PMID 16761576.
42. Parthasarathy M, Gnanadoss JJ. Medium formulation and its optimization to enhance protease production by Streptomyces sp. Isolated from mangroves. Biosci Biotech Res Asia. 2018 Sep 25;15(3):719-28. doi: 10.13005/bbra/2680.
43. Niyonzima FN, More SS. Screening and optimization of cultural parameters for an alkaline protease production by Aspergillus terreus gr. under submerged fermentation. Int J Pharm Biol Sci. 2013;4(1):1016-28 ISSN 0975-6299.
44. Srinivasan MC, Vartak HG, Powar VK, Sutar II. High activity alkaline protease production by a Conodiobolus SP. Biotechnol Lett. 1983 May 1;5(5):285-8. Available from: https://link.springer.com/article/10.1007/BF01141126
45. Abidi F, Limam F, Nejib MM. Production of alkaline proteases by Botrytis cinerea using economic raw materials: assay as biodetergent. Process Biochem. 2008 Nov 1;43(11):1202-8. doi: 10.1016/j.procbio.2008.06.018.
46. Palsaniya P, Mishra R, Beejawat N, Sethi S, Gupta BL. Optimization of alkaline protease production from bacteria isolated from soil. J Microbiol Biotechnol Res. 2012;2(6):695-701. Available from: www.scholarsresearchlibrary.com
47. Laxman RS, Sonawane AP, More SV, Rao BS, Rele MV, Jogdand VV, Deshpande VV, Rao MB. Optimization and scale up of production of alkaline protease from Conidiobolus coronatus. Process Biochem. 2005 Sep 1;40(9):3152-8. doi: 10.1016/j.procbio.2005.04.005.
48. Sathish Kumar R, Ananthan G, Selva Prabhu A. Optimization of medium composition for alkaline protease production by Marinobacter sp. GA CAS9 using response surface methodology – A statistical approach. Biocatal Agric Biotechnol. 2014 Apr 1;3(2):191-7. doi: 10.1016/j.bcab.2013.11.005.
49. Duraikannu D, Chandrasekaran SD. Optimization and modeling studies on the production of a new fibrinolytic protease using Streptomyces radiopugnans_VITSD8. Front Biol. 2018 Feb 1;13(1):70-7. doi: 10.1007/s11515-017-1476-9.
50. Khurana S, Kapoor M, Gupta S, Kuhad RC. Statistical optimization of alkaline xylanase production from Streptomyces violaceoruber under submerged fermentation using response surface methodology. Indian J Microbiol. 2007 Jun 1;47(2):144-52. doi: 10.1007/s12088-007-0028-4, PMID 23100657.
51. Park YS, Kang SW, Lee JS, Hong SI, Kim SW. Xylanase production in solid state fermentation by Aspergillus niger mutant using statistical experimental designs. Appl Microbiol Biotechnol. 2002 May 1;58(6):761-6. doi: 10.1007/s00253-002-0965-0, PMID 12021796.
52. Reddy LV, Wee YJ, Yun JS, Ryu HW. Optimization of alkaline protease production by batch culture of Bacillus sp. RKY3 through Plackett–Burman and response surface methodological approaches. Bioresour Technol. 2008 May 1;99(7):2242-9. doi: 10.1016/j.biortech.2007.05.006, PMID 17596938.
53. Pillai P, Mandge S, Archana G. Statistical optimization of production and tannery applications of a keratinolytic serine protease from Bacillus subtilis P13. Process Biochem. 2011 May 1;46(5):1110-7. doi: 10.1016/j.procbio.2011.01.030.
54. Gouda MK. Optimization and purification of alkaline proteases produced by marine Bacillus sp. MIG newly isolated from Eastern Harbour of Alexandria. Pol J Microbiol. 2006;55(2):119-26. PMID 17419289.
55. De Azeredo LA, De Lima MB, Coelho RR, Freire DM. A low-cost fermentation medium for thermophilic protease production by Streptomyces sp. 594 using feather meal and corn steep liquor. Curr Microbiol. 2006 Oct 1;53(4):335-9. doi: 10.1007/s00284-006-0163-x, PMID 16972130.

56. Mostafa EE, Saad MM, Awad HM, Selim MH, Hassan HM. Optimization conditions of extracellular proteases production from a newly isolated Streptomyces pseudogrisiolus NRC-15. J Chem. 2012 Apr 1;9(2):949-61. doi: 10.1155/2012/168540.
57. Thumar JT, Singh SP. Repression of alkaline protease in salt-tolerant alkaliphilic Streptomyces clavuligerus strain Mit-1 under the influence of amino acids in minimal medium. Biotechnol Bioprocess Eng. 2011 Dec 1;16(6):1180-6. doi: 10.1007/s12257-009-0087-y.
58. Lazim H, Mankai H, Slama N, Barkallah I, Limam F. Production and optimization of thermophilic alkaline protease in solid-state fermentation by Streptomyces sp. CN902. J Ind Microbiol Biotechnol. 2009 Apr 1;36(4):531-7. doi: 10.1007/s10295-008-0523-6, PMID 19152015.
59. Patel R, Dodia M, Singh SP. Extracellular alkaline protease from a newly isolated haloalkaliphilic Bacillus sp.: production and optimization. Process Biochem. 2005 Nov 1;40(11):3569-75. doi: 10.1016/j.procbio.2005.03.049.
60. Pant G, Prakash A, Pavani JVP, Bera S, Deviram GVNS, Kumar A, Panchpuri M, Prasuna RG. Production, optimization and partial purification of protease from Bacillus subtilis. J Taibah Univ Sci. 2015 Jan 1;9(1):50-5. doi: 10.1016/j.jtusci.2014.04.010.
61. Sivakumar K, Sahu MK, Thangaradjou T, Kannan L. Research on marine Actinobacteria in India. Indian J Microbiol. 2007 Sep 1;47(3):186-96. doi: 10.1007/s12088-007-0039-1, PMID 23100666.
62. Mukhtar S, Zaheer A, Aiysha D, Abdulla Malik K, Mehnaz S. Actinomycetes: A Source of Industrially Important Enzymes. J Proteomics Bioinform;10(12). doi: 10.4172/jpb.1000456.
63. Gençkal H, Tari C. Alkaline protease production from alkalophilic Bacillus sp. isolated from natural habitats. Enzyme Microb Technol. 2006 Aug 2;39(4):703-10. doi: 10.1016/j.enzmictec.2005.12.004.
64. Singh S, Bajaj BK. Agroindustrial/forestry residues as substrates for production of thermoactive alkaline protease from Bacillus licheniformis K-3 having multifaceted hydrolytic potential. Waste Biomass Valorization. 2017 Mar 1;8(2):453-62. doi: 10.1007/s12649-016-9577-2.
65. Benluvankar V, Priya SE, Gnanadoss JJ. Medium Formulation and its optimization for increased protease production by Penicillium sp. LCJ228 and its potential in blood stain removal. J Appl Biol Biotechnol;2016(Jan);4(1):020-6. doi: 10.7324/JABB.2016.40104.
66. Gnanadoss JJ, Devi SK. Optimization of nutritional and culture conditions for improved protease production by Aspergillus nidulans and Aspergillus flavus. J Microbiol Biotechnol Food Sci. 2019 Jan 12;04(6):518-23. doi: 10.15414/jmbfs.2015.4.6.518-523.
67. Kuppusamy A, Kumar KV. Optimization of cholesterol oxidase production and 16S rRNA partial sequence of Bacillus cereus strain KAVK4 isolated from butter. J Appl Pharm Sci. 2016 Jul;6(7):061-6. doi: 10.7324/JAPS.2016.60709.
68. Dutta JR, Dutta PK, Banerjee R. Optimization of culture parameters for extracellular protease production from a newly isolated Pseudomonas sp. using response surface and artificial neural network models. Process Biochem. 2004 Oct 29;39(12):2193-8. doi: 10.1016/j.procbio.2003.11.009.
69. Chauhan B, Gupta R. Application of statistical experimental design for optimization of alkaline protease production from Bacillus sp. RGR-14. Process Biochem. 2004 Oct 29;39(12):2115-22. doi: 10.1016/j.procbio.2003.11.002.
70. BhavnaChauhan, RaniGupta. Application of statistical experimental design for optimization of alkaline protease production from *Bacillus* sp. RGR-14. 2004; 39(12):2115-22. doi: 10.1016/j.procbio.2003.11.002