# Efficient Medium for Protease Production by *Bacillus licheniformis* MZK05M9 Optimized through Response Surface Methodology

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**Abstract**

**Background.** Due to certain limitations, the bioprocess development for protease production needs more convenient and realistic statistical approach instead of conventional optimization technique. For an economic bioprocess with enhanced protease yield, Response Surface Methodology (RSM) based on Central Composite Design (CCD) was employed and evaluated in this study. **Materials and methods.** The fermentation was performed with a mutant strain, *Bacillus licheniformis* MZK05M9 (*Bl*M9) using molasses, urea and $CaCl_2.2H_2O$ as carbon, nitrogen and trace element sources respectively in shake flask. The conditions for fermentation were maintained with temperature, pH and agitation at 37°C, 7.5 and 150 rpm respectively. The required number of trials were determined by investigating each variable (Molasses, Urea and $CaCl_2$) at five levels: -α, -1, 0, +1 and +α through CCD with protease yield as the response function and the interaction effects as well as optimal parameters were obtained by using Minitab software. The significance of the independent variables and their interactions were tested by means of analysis of variance (ANOVA) with a 95% confidence level and 3-D surface plots were developed through RSM. **Results.** Upon 20 trials, the optimum values of the tested variables for maximum alkaline protease production as predicted through CCD and RSM were as 0.63%, 0.16%, and 0.11% (w/v) for Molasses, Urea and $CaCl_2.2H_2O$, respectively. The protease activity in Conventionally Optimized (CO) medium was 410 U/ ml and it was predicted as 463.1 U/ ml for statistically optimized medium. Upon experiments with the optimized medium, the protease activity was estimated as 560 U/ ml which was 36.6% (i.e. 1.36 fold) higher than that of CO medium. **Conclusion.** The efficiency of the enzyme in solubilizing the whole feathers was also assessed which indicated that the enzyme produced with cheap substrates could be utilized as a cost effective and eco-friendly agent in poultry feed formulation, leather processing etc.

Significance | Economic production of protease by statistical approach.

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**Abbreviations:** RSM, Response Surface Methodology; CCD, Central Composite Design; ANOVA, Analysis of Variance; TCA, Trichloroacetic acid; BSA, Bovine Serum Albumin; rpm, Rotation per minute; *Bl*M9, *Bacillus licheniformis* MZK05M9.

## 1. Introduction

Proteases are proteolytic enzymes with the ability to degrade protein by breaking down the hydrogen bonds that bind and keep peptides together into specific foldings. Proteases are vital in terms of their physiological roles and commercial applications

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and a wide range of microorganisms including bacteria, moulds, yeasts and actinomycetes etc produce this enzyme (Akcan & Uyar, 2011). Among bacteria, *Bacillus licheniformis, B. subtilis, B. horikoshii, B. sphaericus, B. furmis, B. alcalophilus* (Adinarayana, Bapi Raju, & Ellaiah, 2004) are considered as the attractive producer of proteases for their industrial feasibility. Bangladesh being one of the leading exporter of leather and textile products, needs to take the advantages of protease very badly to ameliorate the current environment pollution as well as the product quality. At present, protease is commercially being produced by fermentation technology in Bangladesh with a mutant strain, *Bacillus licheniformis* MZK05M9 (*Bl*M9) which exhibited about three fold higher enzyme activity than that of the wild one (Hoq, Siddiquee, Kawasaki, & Seki, 2005; Salaheen, Mamun, Khan, & Hoq, 2015) and kerA gene was also cloned in E. coli BL-21 by recombinant DNA technology for higher yield and feasible downstream processing (Nahar et al., 2016).

In fermentation biotechnology, enzyme productivity is increased by optimizing the media ingredients and improving the strains by mutation or gene cloning (Davati & Najafi, 2013). In order to optimize medium, conventional 'one factor at a time' technique was in vogue which is a laborious, expensive and lengthy process unable to provide a comprehensive view of the system behaviour ignoring the effects of all parameters involved with baffling results and lack of predictability (Gokhale, Patil, & Bastawde, 1991). To avoid such problems, Response Surface Methodology (RSM) has been employed with a series of statistical and mathematical techniques to design an efficient medium (Hajji, Rebai, Gharsallah, & Nasri, 2008; Lakshmi & Hemalatha, 2015, 2016; Saxena & Singh, 2010). RSM is a statistical tool in multivariate systems which fits the studied experimental domain in the theoretical design through a response function (Nazir, Shuib, Kalil, Song, & Hamid, 2018; Sarrai et al., 2016; C. Song, Li, Wang, & Shi, 2016) and is useful for constructing models in which a response of interest being influenced by selected variables with the objective of representing optimal values (Deepak et al., 2008; Liu & Wang, 2007; Montgomery, 2006; Sayyad, Panda, Javed, & Ali, 2007). For more accurate and true response surface, a wide variety of functional forms of second-order models, e.g. Central Composite, Box-Behnken and Doehlert designs, are employed (Adinarayana & Ellaiah, 2002; Carvalho, Serralheiro, Cabral, & Aires-Barros, 1997; Li et al., 2007; Rahman & Gomes, 2003; Srinivas, Chand, & Lonsane, 1994; Xiao, Liu, Qin, & Xu, 2007). Optimization of several bioprocesses, including fermentations (Sen, 1997) and enzyme immobilization techniques (Chang, Chang, Yen, & Shieh, 2007; Z. Song et al., 2007) etc. involve RSM and experimental designs to save cost and time along with improved productivity and reduced process variability (Rao, Kim, & Rhee, 2000).

The alkaline protease production potential of the mutant strain *Bl*M9 in commercial media and various cheap substrate especially soybean meal based media was investigated employing RSM and CCD (Mamun, Mian, Saifuddin, Khan, & Hoq, 2017). With a view to avoiding probable complexities during downstream processing of enzyme due to soybean meal and developing a more cost effective bioprocess, molasses, urea and $CaCl_2$ based medium was proposed and optimized involving RSM and CCD. Whether the optimized medium obtained through statistical approach supports better enzyme productivity or not was also evaluated in this study.

## Materials and methods

**Bacterial strain.** A mutant bacterial strain *Bacillus licheniformis* MZK05M9 (*Bl*M9) obtained from the Enzyme and Fermentation Biotechnology Laboratory, Department of Microbiology, University of Dhaka, was used in this study.

**Inoculum preparation.** 5 ml of Tryptone Soy Broth (TSB) medium (Sigma, USA) [pH 7.5] prepared in a test tube was autoclaved and 1 single colony of *Bl*M9 mutant strain from Tryptone Soy Agar was inoculated aseptically in that medium. Upon overnight incubation at 37 °C, fresh bacterial culture was used everytime as inoculum in further fermentation process.

**Medium optimization through statistical approach.** The amounts (%) of Molasses, Urea (Merck, Germany) and $CaCl_2.H_2O$ (Sigma, USA) required for maximum enzyme yield were optimized through the statistical program Response Surface Methodology (RSM). A Central Composite Design (CCD) with three independent variables i.e. Molasses, Urea and $CaCl_2.H_2O$ was applied to determine the required number of trials and the amounts of variables through the factorial design.

A total of 20 experiments was found to be sufficient to calculate the coefficients of the second-order polynomial regression model for the three variables. Each variable was investigated at five levels, i.e. -α, -1, 0, +1 and +α, as shown in Table 1 where the highest and lowest values of the variables were considered from the previous reports (Abinaya, Ramya, Sivakami, Ponnusami, & Sugumaran, 2017; Huang, Badger, Haney, & Evans, 2007; Qureshi, Bhutto, Khushk, & Dahot, 2011; Suganthi et al., 2013). The enzyme yield is thus explained by the following empirical second order polynomial model.

$$Y=\beta_0+\beta_1X_1+\beta_2X_2+\beta_3X_3+\beta_{11}X_1^2+\beta_{22}X_2^2+\beta_{33}X_3^2+\beta_{12}X_1X_2+\beta_{13}X_1X_3+\beta_{23}X_2X_3 \quad \text{..... (1)}$$

Here, Y is the enzyme yield, $\beta_0$ is the interception coefficient, $\beta_1$, $\beta_2$ and $\beta_3$ are linear coefficients; $\beta_{11}$, $\beta_{22}$ and $\beta_{33}$ are quadratic terms; $\beta_{12}$, $\beta_{13}$ and $\beta_{23}$ are interaction coefficients and $X_1$, $X_2$ and $X_3$ are coded independent variables studied.

The analyses were carried out in triplicate and the statistical analysis was performed using the Minitab software (Version 17). Data were analyzed by the analysis of variance (ANOVA), and p-value lower then 0.05 was considered significant in surface response analysis. The optimal values of the operation parameters

Table 1 | **Level of independent variables established according to central composite design (CCD).**

| Independent variables | High (+1) | Low (-1) | Mean (0) | +α | -α |
|---|---|---|---|---|---|
| Molasses | 1 | 0.5 | 0.75 | 1.17 | 0.33 |
| Urea | 0.2 | 0.1 | 0.15 | 0.234 | 0.066 |
| $CaCl_2.2H_2O$ | 0.1 | 0.05 | 0.075 | 0.117 | 0.033 |

Table 2 | **Experimental designs of the five levels and the respective experimental results as well as predictive values.** ***EA: Enzyme activity which indicates the enzyme yield.**

| Trials | Molasses | Urea | $CaCl_2.2H_2O$ | Experimental *EA (U/ml) | Predicted EA (U/ml) | Residuals |
|---|---|---|---|---|---|---|
| 1 | 0.5 | 0.1 | 0.05 | 320 | 333.035 | -13.0352 |
| 2 | 1 | 0.1 | 0.05 | 397 | 398.268 | -1.2678 |
| 3 | 0.5 | 0.2 | 0.05 | 450 | 432.516 | 17.4837 |
| 4 | 1 | 0.2 | 0.05 | 420 | 440.749 | -20.7489 |
| 5 | 0.5 | 0.1 | 0.1 | 430 | 410.042 | 19.9580 |
| 6 | 1 | 0.1 | 0.1 | 402 | 420.275 | -18.2746 |
| 7 | 0.5 | 0.2 | 0.1 | 455 | 454.523 | 0.4769 |
| 8 | 1 | 0.2 | 0.1 | 420 | 407.756 | 12.2443 |
| 9 | 0.33 | 0.15 | 0.075 | 360 | 375.177 | -15.1771 |
| 10 | 1.17 | 0.15 | 0.075 | 407 | 390.704 | 16.2956 |
| 11 | 0.75 | 0.066 | 0.075 | 390 | 382.878 | 7.1224 |
| 12 | 0.75 | 0.234 | 0.075 | 450 | 456.004 | -6.0039 |
| 13 | 0.75 | 0.15 | 0.033 | 432 | 421.935 | 10.0647 |
| 14 | 0.75 | 0.15 | 0.117 | 450 | 458.946 | -8.9462 |
| 15 | 0.75 | 0.15 | 0.075 | 458 | 449.365 | 8.6347 |
| 16 | 0.75 | 0.15 | 0.075 | 454 | 449.365 | 4.6347 |
| 17 | 0.75 | 0.15 | 0.075 | 444 | 449.365 | -5.3653 |

estimated by the three-dimensional response surface analysis of the independent variables (Molasses, Urea and $CaCl_2$.H2O), the range and levels of which are mentioned in Table 1, and the dependent variable (Y).

**Production of Alkaline Protease in shake Flask.** The amount (%) of molasses, urea and $CaCl_2$ for the trials was determined by the factorial design for variable optimization as specified in Table 2. These varying amounts of ingredients were used accordingly to prepare the media of different compositions for protease production in 500 mL Erlenmeyer flask and the pH was maintained at 7.5. The media upon sterilization were inoculated with the mutant, *Bl*M9 and in all cases, the required amount of inoculum was standardized so that the incubation starts at an OD600nm of 0.1. The flasks were then kept in an orbital shaking incubator (New Brunswick™ Excella® E25, USA) at 37°C and 150 rpm.

**Protease assay.** Protease activity was determined according to the modified method of Kreger and Lockwood (Mamun et al., 2017). In brief, 400ul of 1% Azo-casein (Sigma, USA) solution in 0.05 M Tris -HCI buffer [pH 8.5] (Sigma, USA) was mixed with 400ul of culture supernatant and kept for 1 hour at 37°C. The reaction was then stopped by adding 135 ul of 35% trichloroacetic acid (TCA) (BDH, England) and the mixture was kept at 4°C for 2- 3 min. Upon centrifugation at 13,000 rpm for 10 min, 0.75 ml of the supernatant was mixed with 0.75 ml of 1.0 M NaOH (Merck, Germany) and the absorbances taken at 440 nm were recorded immediately against the control, prepared initially by adding TCA into the culture supernatant to inhibit the enzyme activity. One unit of protease activity was considered as the amount of enzyme that produces an increase of 0.01 in absorbance under the above assay conditions.

**Estimation of extra cellular protein concentration.** The extracellular soluble protein in the culture supernatant was estimated following Bradford method and using Bovine Serum Albumin (BSA) (Sigma, USA) as a standard (Ernst & Zor, 2010).

## Results and Discussion

**Medium ingredients for protease production.** With a view to economic production of protease by mutant *Bl*M9, molasses, a byproduct of sugar industry, and urea were chosen as carbon and nitrogen source respectively. Molasses has successful history of use as an inexpensive sole carbon source for the production of many microbial enzymes (El-Enshasy, Mohamed, Farid, & El-Diwany, 2008; Helal, Amer, & Abdelwahed, 2012; Mourin, Shishir, Khan, & Hoq, 2015; Qureshi et al., 2011) and urea was reported to provide excellent support as nitrogen source in many cases (Abinaya et al., 2017; Aksoy, Uzel, & Hameş Kocabaş, 2012; Huang et al., 2007; Suganthi et al., 2013). On the other hand, divalent metal ions are required in the fermentation medium for optimum production of alkaline protease, protection of enzymes from conformational changes and to regulate the enzyme activity positively. Calcium derivative i.e. $CaCl_2$, for such roles and inducing as well as stabilizing capacities for many enzymes (Bhunia, Basak, & Dey, 2012; Sharma, Kumar, Panwar, & Kumar, 2017) was chosen as mineral source. The selection of cheap substrates could thereby result in a reduction of approximately 30% in the production cost of protease enzyme as compared to other previous studies (Hoq et al., 2013; Mamun et al., 2017; Md. Mahmuduzzaman Mian, 2014).

Optimization of the amount of ingredients for maximum protease yield is a prerequisite in bioprocess development for which a statistical approach, Response Surface Methodology was employed instead of conventional one variable at a time technique which usually fails to consider the effects of all the parameters involved. RSM is a combination of statistical and mathematical methods to select the best experimental conditions employing the lowest number of experiments in order to get appropriate results (Arslan-Alaton, Tureli, & Olmez-Hanci, 2009; Sarrai et al., 2016).

RSM Model Development

The effects of three variables including Molasses, Urea and $CaCl_2.2H_2O$ on the protease enzyme production were selected as

Table 3 | **ANOVA for the response surface quadratic model. $R^2$ = 0.8879, Adjusted $R^2$= 0.7870**

| Source | Df | Seq SS | Contribution | Adjusted SS | Adjusted MS | F value | *p*-value |
|---|---|---|---|---|---|---|---|
| **Model** | 9 | 21987.5 | 88.79% | 21987.5 | 2443.05 | 8.80 | 0.001 |
| **Linear** | 3 | 8399.5 | 33.92% | 8399.5 | 2799.83 | 10.09 | 0.002 |
| **A** | 1 | 291.0 | 1.18% | 291.0 | 291.03 | 1.05 | 0.330 |
| **B** | 1 | 6454.9 | 26.07% | 6454.93 | 6454.93 | 23.26 | 0.001 |
| **C** | 1 | 1653.5 | 6.68% | 1653.5 | 1653.51 | 5.96 | 0.035 |
| **Square** | 3 | 8938.5 | 36.10% | 8938.5 | 2979.49 | 10.74 | 0.002 |
| **A*A** | 1 | 7260.9 | 29.32% | 7948.2 | 7948.23 | 28.64 | 0.000 |
| **B*B** | 1 | 1534.1 | 6.20% | 1613.1 | 1613.13 | 5.81 | 0.037 |
| **C*C** | 1 | 143.5 | 0.58% | 143.5 | 143.48 | 0.52 | 0.489 |
| **2 way interaction** | 3 | 4639.5 | 18.78% | 4649.5 | 1549.83 | 5.58 | 0.016 |
| **A*B** | 1 | 1624.5 | 6.56% | 1624.5 | 1624.50 | 5.85 | 0.036 |
| **A*C** | 1 | 1512.5 | 6.11% | 1512.5 | 1512.50 | 5.45 | 0.042 |
| **B*C** | 1 | 1512.5 | 6.11% | 1512.5 | 1512.50 | 5.45 | 0.042 |
| **Error** | 10 | 2775.5 | 11.21% | 2775.5 | 277.55 | | |
| **Lack of Fit** | 5 | 2554.2 | 10.31% | 2554.2 | 510.83 | 11.54 | 0.009 |
| **Pure Error** | 5 | 221.3 | 0.89% | 221.3 | 44.27 | | |
| **Total** | 19 | 24763.0 | 100.0% | | | | |

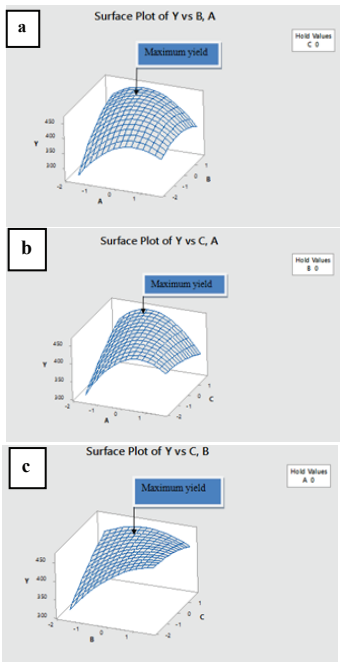


Figure 1 | **Effect of different variables on protease production determined through response surface methodology. Varibales are in a) Molasses and Urea, b) Molasses and $CaCl_2.2H_2O$, c) Urea and $CaCl_2.2H_2O$ [Here, A=Molasses, B=Urea, C=Ca-$Cl_2.2H_2O$ and Y= Enzyme Yield].**

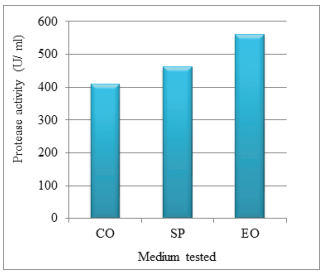


Figure 2 | **Comparison of protease yields among the media in shake flask culture. (CO: Conventionally optimized, SP: Software predicted and EO: Experimentally obtained).**

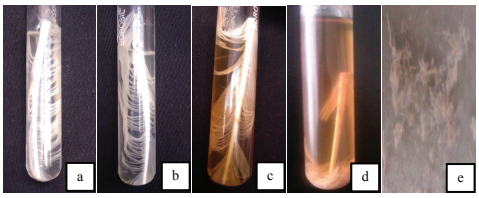


Figure 3 | **Hydrolysis test of chicken feather. Feather of a) 0 day and b) 7th day treated without enzyme (control). Feather of c) 0 day, d) 7th day and e) 14th day treated with the enzyme.**

factors in the Central Composite Design. As a response, the protease enzyme activity was chosen and a total of 20 experiments were employed for the response surface modeling (Table 2) where the order of experiments was arranged randomly. The observed and predicted results for the enzyme activity are also recorded in Table 2. The coefficients of the second-order fitting equation was to calculated using Minitab software (Version 17) and the ANOVA test was employed to assess the suitability of the models. Thus the second-order polynomial equation could be expressed by Equation (2) (conf. Equation (1)):

$$\text{Protease Yield, } Y = 449.37 + 4.62X_1 + 21.74X_2 + 11.00X_3 - 23.48\,X_1{}^*\,X_1 - 10.58\,X_2{}^*\,X_2 - 3.16\,X_3{}^*\,X_3 - 14.25\,X_1{}^*\,X_2 - 13.75\,X_1{}^*\,X_3 - 13.75\,X_2{}^*\,X_3 \quad \ldots\ldots\ldots\ldots\ldots (2)$$

Where $X_1$ is Molasses, $X_2$ is Urea and $X_3$ is $CaCl_2.2H_2O$.

According to the monomial coefficient value of regression model Equation (2), $X_1$ = 4.62 (Molasses), $X_2$ = 21.74 (Urea) and $X_3$ = 11.00 ($CaCl_2.2H_2O$), and the order of priority among the main effect of impact factors is Molasses (X1) > $CaCl_2.2H_2O$ ($X_3$) > Urea ($X_2$).

## Statistical Analysis

The results of the analysis of variance (ANOVA) as summarized (Table 3), implies the soundness of the model. ANOVA subdivides the total variation in a set of data into component parts associated with specific sources of variation in order to test the hypotheses on the parameters of the model (Sarrai et al., 2016). The statistical significance in all analyses was determined at a 95% confidence level ($\alpha$ = 0.05). Various descriptive statistics such as the p-value, F-value, and the degree of freedom (df) were used to assess the results; the determination coefficient ($R^2$) of each coefficient in Equation (2) was determined by Fisher's F-test and values of probability >F. As shown in Table 3, a small probability value ($p < 0.001$) indicates that the model was highly significant and could be used to predict the response function accurately. The coefficients of determination $R^2$ (correlation coefficient) and adjusted coefficients of determination $R^2$adj were used to evaluate the Goodness-of-fit for the model. The larger the value of the correlation coefficient, the higher the reliability of the model in predicting the response. Here in this study, the $R^2$ = 0.8879 indicated that 88.79% of the response variability could be explained by the model for the protease enzyme yield as a response.

## Effects of model parameters and their interactions

The significance of each model parameter was determined by means of Fischer's F- value and p-value. The F- value is the test for comparing the curvature variance with residual variance and probability >F (p-value) is the probability of seeing the observed F- value if the null hypothesis is true. Since small probability values call for rejection of the null hypothesis and the curvature is

not significant, the larger the F- value and the smaller the p-value, the more significant the corresponding coefficient is (Bayraktar, 2001). It was concluded that one of the independent variables of the quadratic model, urea is highly significant parameters as $p < 0.001$ (Table 3). Moreover, linear terms of $CaCl_2.2H_2O$ and the square effects, A*A, B*B and the combination of A*B, A*C, B*C are significant because $p < 0.05$ (Table 3). Likewise the linear terms of A and the square effects of C*C are insignificant (Where A= Molasses, B= Urea and C= $CaCl_2.2H_2O$).

The Minitab software was used to genrate three-dimensional (3D) response surfaces which are graphical representations of the regression equation for the optimization of amounts of medium ingredients for maximum protease yield. In these plots, the response functions of two factors are presented while all other factors are at the fixed levels. The results of the interactions between three independent variables and the dependent variable are shown in Figure 1.

The individual and mutual effects of Molasses and Urea on the protease yield as depicted in the surface plot (Figure 1) revealed that the highest yield was attained when Urea level was high and Molasses level was low. Again in Figure 1b, the individual and mutual effects of Molasses and $CaCl_2.2H2O$ on the protease yield as shown by the surface plot indicates that the highest yield was obtained when $CaCl_2.2H_2O$ level was high and Molasses level was low. Similarly, Figure 1c shows the individual and mutual effects of Urea and $CaCl_2.2H_2O$ on the protease yield. The relevant surface plot demonstrates that the highest yield is obtained when both Urea and $CaCl_2.2H_2O$ levels are high but $CaCl_2.2H_2O$ has greater effect than that of Urea. Finally it could be concluded that, molasses, urea and $CaCl_2.2H_2O$ had a positive effect on protease yield.

## Evaluation of the software optimized medium

The maximum enzyme activity i.e. the enzyme yield as predicted by the software Minitab for the optimized medium was 463.1 U/ml and the composition i.e. Molasses- 0.6353%, Urea- 0.1627% and $CaCl_2.2H_2O$- 0.1170% for the respective medium was optimized by regression model. The efficiency of the optimized medium was assessed in shake flask fermentation following protease production by the strain *Bl*M9 and the protease yield was estimated as 560 U/ ml (Figure 2). It could be observed that the protease yield was increased to 1.3 fold than that (410 U/ ml) of conventionally optimized medium.

Protease yields were improved through such statistical approaches as reported previously e.g. 14.0 fold increase (final activity 770.66 U/ mL) by *Aspergillus clavatus* ES1 through RSM & Plackett-Burman design (Hajji et al., 2008), 1.4 fold increase (final 185.4 U/ mL) in basal medium by *Bacillus licheniformis* through RSM & CCD (Lakshmi & Hemalatha, 2016), 1.24 fold increase (final 205±0.35 U/ mL) in basal medium *Bacillus cereus* strain S8 through RSM & CCD (Lakshmi & Hemalatha, 2015) and 1.75 fold increase (final 577 U/ mL) in glucose-soybean medium by *Bacillus* sp. through RSM & CCD (Saxena & Singh, 2010). Interestingly, although the medium optimized in this study is composed of very cheap substrates, it demonstrated highly comparable protease yield.

Beside azo-casein digest method, protease activity of culture supernatant was further evaluated by feather digest method in test tubes in parallel to control i.e. uninoculated broth. Degradation of feather was not observed in control case up to 14 days whereas after 7 days, feather was completely digested by the culture supernatant containing the secreted extracellular protease (Figure 3). Upon 60% ammonium sulfate precipitation, the crude enzyme was concentrated and a 3.025 fold increase in specific activity was obtained. Further ultrafiltration by centricon-100 (Roti spin, Carl Roth, Germany) caused an increase of 16.59 folds in specific activity (Data not shown) which suggested about an effective mean for downstream processing i.e. purification of the enzyme through cross flow velocity ultrafiltration would be a correct choice.

## Conclusion

The enzyme yield was improved by involving the statistical approach, RSM & CCD, instead of conventional method and previous studies also recommended this upgradation in bioprocess technology. Molasses, Urea and $CaCl_2$ based medium thus optimized would be economic reducing the costs both in upstream and downstream processing which in turn, will facilitate industrial production and application of proteases in Bangladesh.

## Author contributions

MMM designed the research, performed the experiments and drafted the manuscript. AAM supervised the experiments and evaluated the data as well as results of the manuscript meticulously. SNK and MMH supervised the whole research and reviewed the manuscript critically.

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## Competing financial interests

Authors have declared that no competing interest exist.

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