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Response Surface Methodology for the Optimization of Proteases Production by a Novel Egyptian Isolate *Bacillus amyloliquefaciens* 35s

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Authors' contributions

This work was carried out in collaboration between all authors. All authors read and approved the final manuscript.

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ABSTRACT

Aims: The present work aimed to optimize proteases production by *Bacillus amyloliquefaciens* 35s using the response surface methodology (RSM).
Study Design: Variables affecting proteases production were screened using a Plackett–Burman design. Face Centered Central Composite Design (FCCCD) of RSM was adopted for the augmentation of total proteases production assessed at three coded levels (–1, 0, +1). All obtained data were analyzed by ANOVA with post hoc multiple comparison analysis performed using Tukey's HSD.
Place and Duration of Study: Department of Agricultural Microbiology, Faculty of Agriculture, Ain Shams University, between March 2014 and September 2014.
Methodology: *Bacillus amyloliquefaciens* 35s was used for proteases production. Modified TGY (Tryptone gluscose yeast extarct) medium was the basal medium. Impacts of nutritional factors (carbon and nitrogen and mineral salts) were studied using Plackett-Burman design with fold over augmenting method. "Design Expert® 8.0.7.1" Stat-Ease was used to analyze the experimental

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Plackett–Burman design. Temperature, pH and agitation rate (using shake flask) were optimized statistically by the factorial FCCCD of the RSM. Validation of statistical model of physical factors was done by carrying out the experiment at optimum conditions of the process parameters as determined from the model. Optimum conditions obtained through RSM in terms of FCCCD were examined and verified in a 5 L bench top continuous stirred tank bioreactor and production process was scaled-up in a batch process with controlled and non-controlled pH. Fermented medium was centrifuged to collect cells and determination of biomass and protease concentration.

Results: Among the significant media components, peptone and starch showed to have significant effects on the response as for protease production, with confidence level > 98% and were further optimized using FCCCD. Conditions promoted proteases production were different from those enhanced cell growth. Physical parameters indicated that production of proteases by *Bacillus amyloliquefaciens* is non-growth dependent. Maximum proteases production predicted (992.12 u/ml) was observed near the mid-point (0) values (concentrations) of both peptone (10 g/l) and starch (10 g/l) and the experimental value 935 u/ml was very close to the predicted value validating the model. The final proteases production in the bioreactor reached 1530 u/ml obtained within 12-14 h at 0.6 vvm aeration and 120 rpm of agitation speed.

Conclusion: Instead of conventional method of one variable at time approach, Response Surface Methodology, as statistical approach, showed to be adequate and efficient to optimize protease production by *Bacillus amyloliquefaciens*.

Keywords: Proteases production; statistical optimization; response surface methodology (RSM); face centered central composite design (FCCCD); Bacillus amyloliquefaciens 35s; bioreactor.

1. INTRODUCTION

The inability of plant and animal proteases to meet current world demands has led to an increased interest in microbial proteases. Microbial proteases are preferred over those from plant and animal sources since they possess almost all the characteristics desired for their biotechnological applications. Bacterial proteases are increasingly studied due to its importance and subsequent applications in industry and biotechnology [1]. Each organism used for proteases production has its own requirement of special conditions for maximum enzyme production [2]. The optimization of medium composition for proteases production is done to maintain a balance between the various medium components [3]. Approximately 90 % of the industrial enzymes are produced using submerged fermentation (SmF) [4]. The classical method of experimental optimization "one-variable-at-a-time-approach" involves changing one variable at a time keeping the others constant is the most frequently used operation to obtain maximum cell density, high yields of the desired metabolic product, or enzyme levels in microbial system. This approach is not only time consuming, but ignores the combined interactions among various physicochemical parameters [5]. Oppositely, the statistical approach using response surface methodology (RSM) developed by Box et al. [6], is a collection of mathematical and statistical techniques that are useful for modeling and analysis; in applications where, a response of interest is influenced by several variables and the objective is to optimize this response [7]. The response surface approach involving a face-centered central composite design (FCCCD) was adopted for improving proteases production by the *Bacillus* sp. RGR-14 [8]. Ramnani et al. [9] used the FCCCD to improve the production of proteases and bio surfactant by *B. licheniformis*RG1. The effects of soybean meal, maltose 50, Tween 80 and initial pH on proteases production were evaluated using RSM by Tari et al. [10].

The central composite design (CCD) of RSM was used in the case of *Bacillus* sp. RKY3 by Reddy et al. [11] for improving proteases production. The three variables, i.e. sucrose, yeast extract and KNO_3, which had played a significant role in enhancing the production of alkaline proteases, were optimized with response surface methodology [12]. CCD was applied for the optimization studies of media parameters for extracellular proteases production by *Halobacterium* sp. SP1(1) [13]. Zhou et al. [14] in their study optimized the mycelial biomass and proteases production by *Laccocephalum mylittae* in submerged fermentation using RSM and orthogonal matrix method. Rai and Mukherjee [15] have used response surface methodology to attain an optimum proteases production of 518 U by *B. subtilis*DM-04 in submerged fermentation.

Manikandan et al. [16] carried out the optimization of growth medium for proteases production by *Haloferax lucentensis* VKMM 007 with help of RSM.

To scale up batch microbial production of proteases, several strategies have been implemented to obtain high yields of proteases in a fermenter. Controlled batch fermentations were used for improving proteases production using a number of microorganisms [1,17,18,11].

The aim of this work was to optimize the nutritional requirements and the cultivation conditions influencing microbial production of proteases by the novel Egyptian strain *Bacillus amyloliquefaciens* 35s and to study the effect of different factors interactions using response surface methodology.

2. MATERIALS AND METHODS

2.1 Microorganism

A novel proteolytic strain (*Bacillus amyloliquefaciens* 35s), was used in this investigation for proteases production. This strain was isolated in a previous work from soil of delta Egypt and identified according to the 16S rRNA gene sequencing analysis [19]. Culture was maintained on nutrient agar slants at 4°C and sub-cultured at monthly intervals. Nutrient agar medium [20], was used for bacterial stock culture maintenance and standard inoculum preparation. It has the following composition (g/l of distilled water): meatextract 3, peptone 5, pH 7. Standard inoculum was prepared by inoculating 50 ml of nutrient broth medium in 250 ml Erlenmeyer flasks with a full loop of tested culture. The inoculated flask was incubated on a rotary shaking incubator (Lab-line Ltd.) at the rate of 120 rpm for 24 h at 30°C and considered as the standard inoculum (7.0 x 10^5/ml viable cells) for shake flasks and bioreactor experiments.

2.2 Optimization of Proteases Production

The efficient proteases producing isolate was used in this experiment and thereafter. All experiments were all run in triplicates and average response was taken, and modified TGY medium [19] was used as the basal medium, which has the following composition (g/l): Starch, 10 and Peptone 10. Unless otherwise mentioned, all of the following experiments were run in 50 ml of tested medium in 250ml Erlenmeyer flasks, and after sterilization, flasks were inoculated with 5 ml of 24 h culture of standard inoculum. Media were adjusted to pH 7.0 and flasks were incubated at 30°C on a rotary shaking incubator at 120 rpm. Samples of 10 ml were taken after 24 h of incubation and used for determination of biomass formation and proteases concentration.

2.2.1 Statistical screening of physical factors using spss software

Data of "one variable at a time" approach experiment [19] on temperature, pH and agitation speed was analyzed statistically using one-way ANOVA with post hoc multiple comparison analysis using Tukey's HSD [21].

2.2.2 Statistical screening of nutritional factors using Plackett-Burman (PB) design

Impacts of nutritional factors, including carbon and nitrogen sources and mineral salts, were studied using Plackett-Burman design for 24 trials with semi fold over augmenting method, which doubles the number of runs to increase the resolution of the design. "Design Expert® 8.0.7.1" Stat-Ease, Inc., Minneapolis, USA, was used to analyze the experimental Plackett–Burman design. Five components were selected for the study with each variable being represented at two levels, high (+1) and low (-1) (Table 1). The variables chosen for the present study were peptone, starch, $CaCl_2$, $MgSO_4$ and KH_2PO_4. The experimental design with the name, symbol code, and actual level of the variables is shown in Table 2. After inoculation, flasks were incubated in a shaker incubator (Lab line Ltd.) at 37°C with shaking at 120 rpm. The growth measurements and proteases production assays was done after 24 h of incubation.

2.2.3 Optimization of physical factors using Face Centered Central Composite Design (FCCCD) of Response Surface Methodology (RSM)

Based on the results obtained from traditionally variable approach, pH, temperature and agitation rate were selected for the study of RSM. The process parameters were optimized statistically using the full factorial Face Centered Central Composite Design (FCCCD) of the Response Surface Methodology (RSM) by isolate 35s. The software "Design Expert" (Version 8.0.7.1, Stat-Ease Inc., Minneapolis, USA) was used to design the experiment, data analysis and the quadratic

model building. A 20 full factorial FCCCD, with six axial points and six replications at the center points (n_0=6) leading to a total number of 20 experiments was carried out for the optimization process with a central coded value for each variable which is considered as zero. The minimum and maximum ranges of variables investigated and the full experimental plan with respect to their values in actual and coded form are listed in Tables 3 and 4, respectively. Upon completion of experiments, the average maximum proteases production was taken as the dependent variable or response (Y). A second order polynomial equation was then fitted to the data by multiple regression procedure. For a three factor system, the model equation was: $Y= \beta_0 + \beta_1A +\beta_2B+ \beta_3C + \beta_{11}A^2+\beta_{22}B^2+ \beta_{33}C^2+ \beta_{12}AB+\beta_{13}AC+\beta_{23}BC$ Where, Y, predicted response;, β_0 intercept; β_1, β_2, β_3, linear coefficients; β_{11}, β_{22}, β_{33}, squared coefficients; β_{12}, β_{13} , β_{23}, interaction coefficients. The statistical significance of the model equation and the model terms were evaluated via Fisher's test. The quality of fit of the second-order polynomial model equation was expressed via the coefficient of determination, R^2, and the adjusted R^2. The fitted polynomial equation was then expressed as three-dimensional surface plots to illustrate the relationship between the responses and the experimental levels of each of the variables utilized in this study, as mentioned by Reddy et al. [11].

2.2.4 Optimization of nutritional factors using Face Centered Central Composite Design (FCCCD) of Response Surface Methodology (RSM)

Based on the results obtained from the PB design, starch and peptone were selected for the study of RSM and further evaluation for their interactive behaviors using a statistical approach was carried out. The FCCCD of RSM was adopted for the augmentation of total proteases production assessed at three coded levels (–1, 0, +1) (Table 5). A total of 13 runs were employed with five replicates at the center point. All variables were taken at a central coded value that was defined as zero. The coded and actual values of the variables (peptone and starch) at various levels and their interactions are given in Table 6.After inoculation, flasks were incubated in a shaker incubator (Lab line Ltd.) at 37°C at 120 rpm. The growth measurements and proteases production assays was done after 24 h of incubation. The data obtained from the RSM on proteases production were subjected to analysis of variance (ANOVA). After running the experiments and measuring the activity levels, the experimental results of RSM were fitted with the response surface regression procedure using the following second-order polynomial equation: $Y= \beta_0 + \beta_1A +\beta_2B + \beta_{11}A^2 + \beta_{22}B^2+ \beta_{12}AB$ Where Y is the response; β_0 intercept; β_1 and β_2 linear coefficients; β_{11} and β_{22} squared coefficients; and β_{12} interaction coefficients. The statistical significance of the model equation and the model terms were evaluated via Fisher's test. The quality of fit of the second-order polynomial model equation was expressed via the coefficient of determination, R^2, and the adjusted R^2. The fitted polynomial equation was then expressed as three-dimensional surface plots to illustrate the relationship between the responses and the experimental levels of each of the variables utilized in this study.

2.3 Validation of Physical and Nutritional Parameters for Proteases Production Optimization by Isolate 35s

The validation of statistical model of physical factors was done by carrying out the experiment at optimum values of the process parameters (temperature, pH and agitation) as determined from the model. Medium was prepared by adjusting pH to 8. After inoculation, flasks were incubated at 37°C with shaking at 120 rpm. After 24 h, growth and proteases were determined. The experimental values obtained were compared to the values predicted by the model. The validation of statistical model for nutritional factors was done by carrying out the experiment at optimum values of media components (starch and peptone) as determined from the model. To validate the model, the final composition of medium was prepared in triplicates in which 50 ml of media was introduced in 250 ml flasks and sterilized. Pre-inoculum was prepared and inoculation was done as mentioned previously. After inoculation, flasks were incubated at 37°C with shaking speed at 120 rpm. After 24 h, the growth and proteases production were determined. The experimental value was compared to the value predicted by the model.

Table 1. Levels of nutritional factors tested in Plackett–Burman design

Variables	Symbols	Coded levels	
		-1 (Low)	+1 (High)
Peptone (g/l)	(A)	5	15
Starch (g/l)	(B)	8	10
K_2HPO_4 (mM)	(C)	0.01	1
$MgSO_4$ (mM)	(D)	0.05	1
$CaCl_2$ (M)	(E)	0.01	1

Table 2. Plackett–Burman design matrix for optimization of media components

Run order	Peptone (A) g/l	Starch (B) g/l	K_2HPO_4 (C) mM	$MgSO_4$ (D)mM	$CaCl_2$ (E)mM	F	G	H	J	K	L
1	+1(15.00)	-1(8.00)	+1(1.00)	+1(1.00)	-1(0.05)	+1	+1	+1	-1	-1	-1
2	-1(5.00)	+1(10.00)	-1(0.01)	+1(1.00)	+1(1.00)	-1	+1	+1	+1	-1	-1
3	-1(5.00)	+1(10.00)	+1(1.00)	+1(0.01)	+1(1.00)	+1	+1	-1	-1	-1	+1
4	+1(15.00)	+1(10.00)	-1(0.01)	+1(1.00)	+1(1.00)	+1	-1	-1	-1	+1	-1
5	-1(5.00)	+1(10.00)	+1(1.00)	+1(1.00)	-1(0.05)	-1	-1	+1	-1	+1	+1
6	-1(5.00)	-1(8.00)	+1(1.00)	-1(0.01)	+1(1.00)	+1	-1	+1	+1	+1	-1
7	-1(5.00)	-1(8.00)	-1(0.01)	+1(1.00)	-1(0.05)	+1	+1	-1	+1	+1	+1
8	+1(15.00)	-1(8.00)	-1(0.01)	-1(0.01)	+1(1.00)	-1	+1	+1	-1	+1	+1
9	-1(5.00)	-1(8.00)	-1(0.01)	-1(0.01)	-1(0.05)	-1	-1	-1	-1	-1	-1
10	+1(15.00)	+1(10.00)	+1(1.00)	-1(0.01)	-10.05)	-1	+1	-1	+1	+1	-1
11	+1(15.00)	+1(10.00)	-1(0.01)	-1(0.01)	-1(0.05)	+1	-1	+1	+1	-1	+1
12	+1(15.00)	-1(8.00)	+1(1.00)	+1(1.00)	+1(1.00)	-1	-1	-1	+1	-1	+1
13	+1(15.00)	+1(10.00)	+1(1.00)	-1(0.01)	+1(1.00)	-1	-1	+1	-1	-1	-1
14	-1(5.00)	+1(10.00)	-1(0.01)	-1(0.01)	-1(0.05)	+1	+1	+1	-1	+1	-1
15	-1(5.00)	-1(8.00)	+1(1.00)	-1(0.01)	-1(0.05)	-1	+1	+1	+1	-1	+1
16	-1(5.00)	+1(10.00)	+1(1.00)	+1(1.00)	-1(0.05)	+1	-1	-1	+1	-1	-1
17	+1(15.00)	-1(8.00)	-1(0.01)	+1(1.00)	-1(0.05)	-1	-1	+1	+1	+1	-1
18	+1(15.00)	-1(8.00)	-1(0.01)	-1(0.01)	+1(1.00)	+1	+1	-1	+1	-1	-1
19	+1(15.00)	+1(10.00)	-1(0.01)	+1(1.00)	-1(0.05)	-1	+1	-1	-1	-1	+1
20	+1(15.00)	-1(8.00)	+1(1.00)	-1(0.01)	-1(0.05)	+1	-1	-1	-1	+1	+1
21	-1(5.00)	-1(8.00)	-1(0.01)	+1(1.00)	+1(1.00)	+1	-1	+1	-1	-1	+1
22	-1(5.00)	+1(10.00)	+1(0.01)	+1(0.01)	+1(1.00)	-1	-1	-1	+1	+1	+1
23	+1(15.00)	+1(10.00)	+1(1.00)	+1(1.00)	+1(1.00)	+1	+1	+1	+1	+1	+1
24	-1(5.00)	-1(8.00)	+1(1.00)	+1(1.00)	+1(1.00)	-1	+1	-1	-1	+1	-1

Factors F, G, H, J, K and L are dummy variables. The letter I is absent by default in the software. The actual values of factors are given in brackets

Table 3. Variables symbol coded levels of face central composite design (FCCCD) optimization experiment

Variable	Symbol	Levels of the variables tested in FCCCD		
		-1 (Low)	0 (Mid)	+1(High)
Agitation (rpm)	(A)	100	120	140
Temperature (^oC)	(B)	35	37	40
Initial pH	(C)	7.5	8.0	8.5

Table 4. FCCCD matrix of the three physical parameters tested for proteases optimization by *B. amyloliquefaciens* 35s

Run order	Coded levels		
	Agitation (rpm)(A)	Temperature (°C)(B)	pH(C)
1	+1 (140.00)	+1 (40.00)	+1 (8.50)
2	0 (120.00)	+1 (41.70)	0 (8.00)
3	+1 (153.64)	0 (37.50)	0 (8.00)
4	0 (120.00)	0 (37.50)	+1 (8.84)
5	-1 (100.00)	-1 (35.00)	-1 (7.50)
6	+1 (140.00)	-1 (35.00)	+1 (8.50)
7	0 (120.00)	0 (37.50)	0 (8.00)
8	0 (120.00)	0 (37.50)	0 (8.00)
9	-1 (100.00)	+1 (40.00)	-1 (7.50)
10	+1 (140.00)	+1 (40.00)	-1 (7.50)
11	-1 (100.00)	-1 (35.00)	+1 (8.50)
12	0 (120.00)	0 (37.50)	0 (8.00)
13	-1 (100.00)	+1 (40.00)	+1 (8.50)
14	0 (120.00)	0 (37.50)	0 (8.00)
15	0 (120.00)	0 (37.50)	0 (8.00)
16	0 (120.00)	0 (37.50)	-1 (7.16)
17	-1 (86.36)	0 (37.50)	0 (8.00)
18	0 (120.00)	0 (37.50)	0 (8.00)
19	0 (120.00)	-1 (33.30)	0 (8.00)
20	+1 (140.00)	-1 (35.00)	-1 (7.50)

The actual values of factors are given in brackets

Table 5. Levels of the variables tested in FCCCD for media components optimization experiment

Variable	Symbol	Levels of the variables tested in FCCCD (g/l)		
		-1(Low)	0 (Mid)	+1(High)
Starch	(A)	8	9	10
Peptone	(B)	5	10	15

Table 6. FCCCD matrix of two nutritional variables optimization experiment

Run order	Coded levels	
	Starch (g/l) (A)	Peptone (g/l) (B)
1	0 (9.00)	0 (7.50)
2	+1 (10.00)	+1 (10.00)
3	0 (9.00)	-1 (7.50)
4	+1 (10.00)	-1 (5.00)
5	-1 (7.59)	-1 (7.50)
6	+1 (10.41)	-1 (7.50)
7	0 (9.00)	-1 (7.50)
8	0 (9.00)	+1 (11.04)
9	0 (9.00)	-1 (3.96)
10	0 (9.00)	-1 (7.50)
11	-1 (8.00)	0 (10.00)
12	0 (9.00)	-1 (7.50)
13	-1 (8.00)	-1 (5.00)

Values in brackets are the actual values of variable

2.4 Bioreactor Experiments

The optimum conditions obtained through RSM in terms of FCCCD were finally examined and verified in a 5 L bench top continuous stirred tank bioreactor (5 L Bioflow 110 Fermenter, New Brunswick Scientific, USA) with 3 liter working volume. All the ingredients at the final optimized amounts obtained from shake flask experiments were dissolved in 3 L of distilled water and transferred into the fermenter. Samples of 10 ml were taken at 2 h intervals for 24 h and

centrifuged at 15000 rpm for 15 min at 4°C. Dissolved oxygen, pH, biomass (cell dry weight), and proteases production were determined at the end of fermentation period. The inoculum was prepared as mentioned in previously section with inoculum size 5% (v/v). The inoculum was aseptically transferred into the fermenter through the inoculum port. Two batches were conducted in bioreactor; the first one was performed under the following conditions: temperature 37°C, pH 8 (as derived from RSM), aeration rate 0.5 vvm and agitation speed 300 rpm. For the control of pH, sterile 1N NaOH and HCl were provided. pH was under automated control and maintained at 7.66 ± 0.1 by the automatic addition of the acid or base. Sterile 10% silicone powder (Himedia Laboratories Limited, Mumbai) was used as antifoam. Air was provided from an oil-free pump (Model OF01080, (Elgi Equipments Limited, Coimbatore) with a working pressure of 8 kg/cm^3). Rotameter controlled the air flow rate into the fermenter vessel. A sterile filter (0.22 µm) was used as a bridge between the tubing from the rotameter and that connected to the air sparger of the fermenter. A second filter (0.22 µm) on the cooler exit gas was placed to prevent microbes from contaminating the laboratory air in case of slight positive pressure occurs. The second batch was done under the same previous conditions but without controlling pH.

2.5 Extraction and Determination of Biomass and Proteases Concentration

For the extraction of crude proteases, the fermented medium was centrifuged at 10000 rpm for 10 min at 4°C to obtain proteases rich broth. Cells were collected to determine cells dry weight. The supernatant was preserved at 4°C for analysis and the assay was done within 24 h [22]. The total protein content of different enzyme preparations was determined by the method of Lowry et al. [23]. Proteases production was determined according to the modified method of Anson [24].

2.6 Proteases Production Parameters

Productivity (P) = Amount of proteases produced (uml^{-1}) / fermentation time (h) = uml^{-1}h^{-1}
Proteases yield coefficient relative to biomass ($Y_{p/x}$) (ug^{-1}) = Amount of proteases produced (uml^{-1}) / amount of biomass (gl^{-1}) [25].

2.7 Statistical Analysis

Data generated from the above experiments were analyzed using one-way ANOVA with post hoc multiple comparison analysis performed using Tukey's HSD. In shake flask experiments, mean of three replicates were compared using SPSS 16.0 for windows at a significance level of $p<0.05$.

3. RESULTS AND DISCUSSION

3.1 Statistical Screening of Physical Factors Using SPSS Software

Our previous experiments [19] aimed at screening physical factors influencing protease production by *Bacillus amyloliquefaciens* 35s using "one variable at a time" approach and statistically analyzed by SPSS software [21]. Results of these experiments revealed that all the tested physical factors (temperature, pH and agitation speed) were significant, in which they affected proteases production both negatively and positively. Results also showed that best values for the tested physical factors for protease production were agitation speed of 120 rpm, temperature of 37°C and pH 8, and going above or below these values had negative effect on protease production. Therefore, temperature, pH and agitation speed were tested for their interactive effects on proteases production using Response Surface Methodology (RSM) in terms of Face Centered Central Composite Design (FCCCD).

3.2 Statistical Screening of Nutritional Factorsby Plackett-Burman Design (PBD)

Five nutritional factors (starch, peptone, K_2HPO_4, $MgSO_4$ and $CaCl_2$) were analyzed by PBD for their effects on proteases production as illustrated in Table 7. The adequacy of the model was determined, where the "Model *F*-value" of 106.92 and low probability value < 0.0500 (Prob. >F) implied that the model was significant. The variables, evidencing statistically significant effects, were screened. Factors, evidencing values of less than 0.05 (Prob. >F), were considered to have significant effects on the response and these factors were peptone and starch. From analysis of values of the regression coefficients of all the factors, it was found that all of them had a positive effect on proteases production. However, based on percentage

Table 7. Plackett–Burman design for media components with the corresponding observed and predicted values of proteases production

Run order	Peptone (A)	Starch (B)	K_2HPO_4 (C)	$MgSO_4$ (D)	$CaCl_2$ (E)	F	G	H	J	K	L	Proteases production (u/ml)	
												Observed	Predicted
1	+1	-1	+1	+1	-1	+1	+1	+1	-1	-1	-1	144.00	125.54
2	-1	+1	-1	+1	+1	-1	+1	+1	+1	-1	-1	104.00	107.88
3	-1	+1	+1	+1	+1	+1	+1	-1	-1	-1	+1	100.00	107.88
4	+1	+1	-1	+1	+1	+1	-1	-1	-1	+1	-1	565.00	595.71
5	-1	+1	+1	+1	-1	-1	-1	+1	-1	+1	+1	120.00	107.88
6	-1	-1	+1	-1	+1	+1	-1	+1	+1	+1	-1	80.00	55.54
7	-1	-1	-1	+1	-1	+1	+1	-1	+1	+1	+1	67.00	55.54
8	+1	-1	-1	-1	+1	-1	+1	+1	-1	+1	+1	140.00	125.54
9	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	60.00	55.54
10	+1	+1	+1	-1	-1	-1	+1	-1	+1	+1	-1	560.00	595.71
11	+1	+1	-1	-1	-1	+1	-1	+1	+1	-1	+1	576.00	595.71
12	+1	-1	+1	+1	+1	-1	-1	-1	+1	-1	+1	138.00	125.54
13	+1	+1	+1	-1	+1	-1	-1	1	-1	-1	-1	850.00	622.96
14	-1	+1	-1	-1	-1	+1	+1	+1	-1	+1	-1	135.00	135.13
15	-1	-1	+1	-1	-1	-1	+1	+1	+1	-1	+1	77.00	82.79
16	-1	+1	+1	+1	-1	+1	-1	-1	+1	-1	-1	130.00	135.13
17	+1	-1	-1	+1	-1	-1	-1	+1	+1	+1	-1	135.00	152.79
18	+1	-1	-1	-1	+1	+1	+1	-1	+1	-1	-1	148.00	152.79
19	+1	+1	-1	+1	-1	-1	+1	-1	-1	-1	+1	545.00	622.96
20	+1	-1	+1	-1	-1	+1	-1	-1	-1	1	+1	130.00	152.79
21	-1	-1	-1	+1	+1	+1	-1	+1	-1	-1	+1	65.00	82.79
22	-1	+1	+1	+1	+1	-1	-1	-1	+1	+1	+1	140.00	135.13
23	+1	+1	+1	+1	+1	+1	+1	+1	+1	+1	+1	560.00	622.96
24	-1	-1	+1	+1	+1	-1	+1	-1	-1	+1	-1	66.00	82.79

Factors F, G, H, J, K and L are dummy variables. The letter I is absent by default in the software

contribution, it was found that the contribution of $CaCl_2$ was negligible, and the same was found for both K_2HPO_4 and $MgSO_4$. Therefore, these ingredients were avoided in the next step of optimization. Among the significant media components, peptone and starch, with confidence level 95%, were further optimized by FCCCD.

3.3 Optimization of Physical Factors Using Face Centered Central Composite Design (FCCCD) of Response Surface Methodology (RSM)

The statistical design approach using RSM was used to study the individual and interactive effects of various process parameters on proteases production by *B. amyloliquefaciens*. The ranges of the independent variables were selected based on the results of the "one variable at a time" strategy [19]. Table 8 summarizes the effects of agitations speed, incubation temperature and medium pH onproteases production of FCCCD experiment for each individual run along with the predicted response. The results of FCCCD were then analyzed by analysis of variance (ANOVA), which gave the following regression equation (in terms of coded factors) of the levels of proteases production (Y) as a function of Agitation (A), pH (B) and temperature (C). Enzyme activity = + 801.81 - 95.97 * A - 42.18 * B + 56.09* C + 24.13* A * B - 28.87* A * C - 97.37* B * C - 99.46* A^2 - 230.10 * B^2 - 260.86* C^2.

ANOVA for the model was performed and analyzed as follows: the model *F*-value was found to be 12.05. High model *F*-value with a very low probability value [(Prob. >F) less than 0.0001] implied that model was highly significant. The linear term A and the quadratic terms A^2, B^2 and C^2 of the model were found to be significant (Prob.> F less than 0.0500) while interactions were found to be 'not significant'. The value of multiple correlation coefficients R^2 was found to be 0.9156, which indicated that the model can explain 91.56% variation in the response. Also,

the model had an "adequate precision value" of 8.9; this suggested the model can be used to navigate the design space. The model showed coefficient of variation (CV), standard deviation, mean and predicted residual sum of squares (PRESS) values of 15% 132.79, 368 and 8.022E+005 respectively. For this model, the 'lack of fit' was found to be "not significant".

The software Design-Expert suggested solutions based on the data generated. The optimum values of the physical parameters and predicted response of the selected solution are given in Table 8. The regression equation is represented in 3 D response surface plots and 2 D contour plots. These results showed that the conditions promoted proteases production were different from those enhanced cell growth, which is in agreement with the observations of Venugopal and Saramma [26].

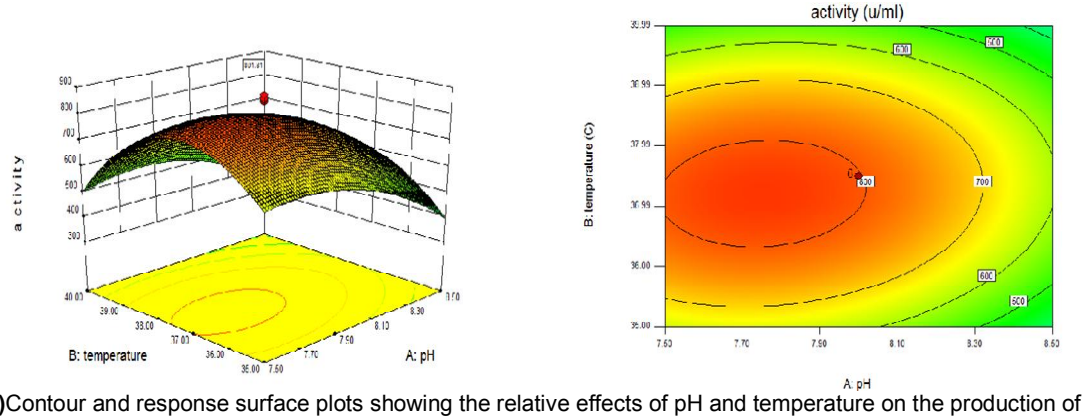
In the present investigation, based on RSM study, various physical parameters and media components showed to have profound effects on proteases production. Analysis of physical parameters indicated that production of proteases by *B. amyloliquefaciens* 35sis non-growth dependent. These results were previously observed by of Genckal and Tari [27]. Yields for different variables can also be predicted from the respective response surface plots (Figs. 1 A-C). Three dimensional (3D) response surface curves were plotted to determine the optimum concentration of each factor for maximum proteases production. Fig. 1 shows the relative effects of four different sets of factors, pH and temperature (A), pH and agitation (B), agitation and temperature (C), while all the other factors were kept at their optimum levels. Results clearly showed a strong degree of curvature of 3D surface, from where the optimum was determined.

3.4 Optimization of nutritional factors using Face Centered Central Composite Design (FCCCD) of Response Surface Methodology (RSM)

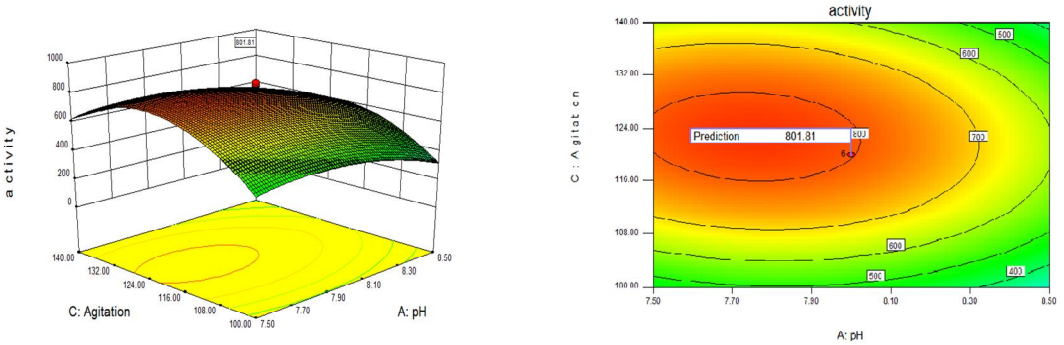
FCCCD of RSM was used to further optimize the two most significant factors determined from the previous screening experiment using PB design, i.e., starch (A) and peptone (B). Table 9summarizes the response (proteases production in terms of proteases activity) for each individual run along with the predicted response of the FCCCD. The results obtained after FCCCD were then analyzed using ANOVA. The RSM gave the following regression equation for the Proteases production Y as a function of starch (A) and peptone (B). Proteases activity Y = +652.60 + 226.59*A + 204.16*B + 166.75 *A *B –112.74*A^2- 145.24*B^2. The results of ANOVA for the model are summarized as follows. The values of F- statistics was found to be 37.67. High Model F-value with a very low probability value (Prob.>F) less than 0.0500 implied that the model was highly significant. The linear terms A and B, the quadratic terms A^2 and B^2 and interaction term AB of the model were found to be significant (Prob.>F) less than (0.0500). In this model, 'A' stands for Peptone and 'B' for starch. The regression equation obtained from analysis of variance (ANOVA) indicated that the multiple correlation coefficient R^2 = 0.9642. Adjusted R^2 and Predicted R^2 values were 0.9386 and 0.8340 respectively. Also, the model has an "adequate precision value" of 17.988 which suggested that the model can be used to navigate the design space. The model showed coefficient of variation (CV), standard deviation, mean and predicted residual sum of squares (PRESS) values of 15.22 %, 75.18, 493.85 and 1.1832E+005 respectively. For this model the 'lack of fit' was found to be 'not significant' (Prob.>F = 0.3130).

The software suggested solutions based on the data generated. The optimum values of the media parameters and predicted response of the selected solution is given in Table 9. Results show the response for the interactive factors peptone and starch. At low concentration of peptone, and higher concentration of starch, there was proportional decrease in proteases production. While at a higher concentration of starch, and increased concentration of peptone, there was a corresponding increase in proteases production. However a further increase in peptone concentration did not result in any proportional increase in production, rather, it caused the production to drop. A simultaneous increase or decrease in the concentration of both peptone and starch resulted in a decrease in production. Maximum predicted proteases production (992.12 u/ml) was observed near the mid-point (0) values (concentrations) of both peptone and starch (both at 10 g/l). There has been a tremendous increase in the use and acceptance of statistical experimental designs in biotechnology. Statistical experimental planning, factorial design and design of experiments investigate the defined input factors to a

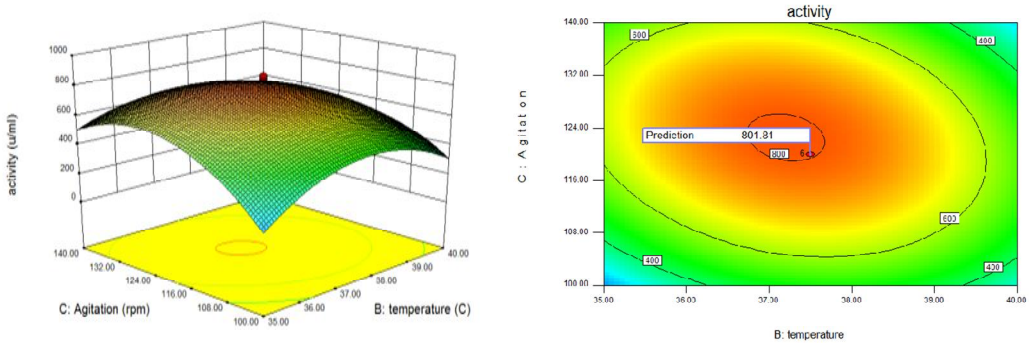
converting system from which mostly common determine the optimum concentration of each factor for maximum proteases production and well-defined output factors or responses, such as product yield and productivity, are generated. The regression equation is represented in 3D response surface plots and 2D contour plots as shown in Fig. 2. The contour plots were elliptical, indicating that the interactions between starch and peptone were significant**,** given that only the significant interactions have been plotted. Three-dimensional (3D) response surface curves were plotted to determine the optimum concentration of each factor for maximum proteases production.



(A) Contour and response surface plots showing the relative effects of pH and temperature on the production of proteases. The agitation speed variable was kept at its optimal levels.



(B) Contour and response surface plots showing the relative effects of pH and agitation speed on the production of proteases. The temperature was kept at optimal levels.



(C) Contour and response surface plots showing the relative effects of temperature and agitation speed on the production of proteases. The pH value was kept at optimal levels.

Fig. 1. 2-dimensional contour plots and 3-dimensional response surface plots showing the effect of physical factors and their interactions on proteases production by *B. amyloliquefaciens* 35s

Table 8. Face-centered central composite design (FCCCD) of the three independent physical factors along with the observed and predicated values of proteases production

Run order	Coded levels			Proteasesproduction(u/ml)	
	Agitation rpm (A)	Temperature (^{o}C) (B)	pH (C)	Observed	Predicted
1	+1	+1	+1	65.00	27.21
2	0	+1	0	196.00	80.06
3	+1	0	0	68.00	158.33
4	0	0	+1	315.00	359.10
5	-1	-1	-1	150.00	191.33
6	+1	-1	+1	334.00	258.08
7	0	0	0	845.00	801.81
8	0	0	0	860.00	801.81
9	-1	+1	-1	174.00	253.47
10	+1	+1	-1	170.00	228.65
11	-1	-1	+1	64.00	8.89
12	0	-1	0	850.00	801.81
13	-1	+1	+1	70.00	167.53
14	0	0	0	855.00	801.81
15	0	0	0	870.00	801.81
16	0	0	-1	731.00	681.89
17	-1	0	0	65.00	30.34
18	0	0	0	530.00	801.81
19	0	-1	0	111.00	221.93
20	+1	-1	-1	650.00	556.01

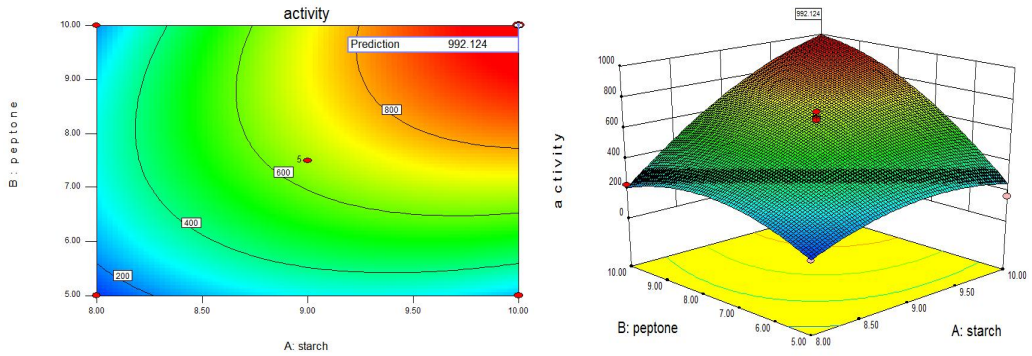


Fig. 2. The two-dimensional contour plot and three-dimensional response surface plot showing the effect of nutritional factors and their interactions on proteases production by *Bacillus amyloliquefaciens* isolate *35s*

The effects of process parameters on proteases production by *B. amyloliquefaciens* 35s were analyzed using ANOVA. The analysis revealed that the quadratic regression model was highly significant as it had a high Fisher's F value (12.05) and a very low probability value (Prob.>F) less than 0.0500). Value of R^2 (multiple correlation coefficient/determination coefficient), which gives measure of how much variability in the observed response was very high for this model (0.9156) which indicated that 91.56 % variation can be explained, while 8.44 % cannot be explained by the model. FCCCD model for nutritional and physical factors was done experimentally to validate the model. Predicted value of 992.12 u/ml was close to the experimental value (935 u/ml, Table 10) which indicates validation of the model.

3.4 Proteases Production by *Bacillus amyloliquefaciens* 35s in Bioreactor as Batch Culture

Scale up of proteases production by *B. amyloliquefaciens* 35s was done in 5L fermenter at 37°C for 24 h in a batch process of controlled

and non-controlled pH. Production medium (3L) was composed of (g/L) starch, 10 and peptone, 10. In non-controlled pH batch culture, modified TGY medium was prepared as previously described in materials and methods section, and pH was adjusted to 7.5, then inoculated and pH was left uncontrolled thereafter. Results of non-controlled pH batch experiment showed that enzyme synthesis increased with increasing medium pH towards alkaline range from neutrality during bacterial growth, maximum production was1505 u/ml achieved at pH 8 and then remained less constant (Fig. 3). In controlled pH batch experiment, pH was adjusted and maintained at 8 as the most effective pH from shake flask experiment [19]. Results of controlled-pH batch experiment showed that proteases production reached 1530 /ml obtained after 12 h at 0.6 vvm, which is 1.6 fold that obtained by shake flasks during batch culture study after 24 h (939 u/ml). It can also be noticed from Fig. 3 that biomass was higher in controlled-pH batch than non-controlled-pH one, and dissolved oxygen was consumed more in controlled-pH batch culture than non-controlled-pH batch.

These results were in agreement with Beg et al. [2] who reported that proteases production by *B. mojavensis* in 14 L bioreactor was 4.2 fold compared to yield obtained by shake flask experiment. They also found that the maximum proteases production was 2398 u/ml, obtained within 10-12 h of incubation compared to 558 u/ml after 24 h in shake flask experiment. Prakasham et al. [28] reported that proteases production reached 9628 u/g biomass in non-controlled pH medium and the pH raised during growth from 7.5 to 9.

Table 9. FCCCD matrix of the two nutritional independent media components along with observed and predicted values of proteases production

Run order	Starch (A)	Peptone (B)	Proteases production (u/ml)	
			Observed	Predicted
1	0	-1	662.00	652.60
2	+1	+1	935.00	992.12
3	0	-1	675.00	652.60
4	+1	-1	166.00	250.31
5	-1	-1	80.00	106.68
6	+1	-1	835.00	747.57
7	0	-1	717.00	652.60
8	0	+1	662.00	650.85
9	0	-1	123.00	73.40
10	0	-1	540.00	652.60
11	-1	0	229.00	205.44
12	0	-1	669.00	652.60
13	-1	-1	127.00	130.63

Table 10. Validation for the FCCCD model of physical and nutritional parameters for optimized proteases production by *B. amyloliquefaciens* 35s

Production parameters (and their levels)	Predicted Activity (u/ml)	Observed activity (u/ml)	Biomass (g/l)	Productivity ($uml^{-1}h^{-1}$)	Productivity yield coefficient relative to biomass (u/g)
Agitation (120 rpm) Temperature (37°C) pH (8.0)	801.81	845	2.3	35.2	367
Starch (10 g/l) Peptone (10 g/l)	992.12	935	1.11	39	374

Productivity (P) = Amount of proteases produced (uml^{-1})/ fermentation time (h) = $uml^{-1}h^{-1}$; Proteases yield coefficient relative to biomass ($Y_{p/x}$) (ug^{-1}) = Amount of proteases produced (uml^{-1}) / amount of biomass (gl^{-1})

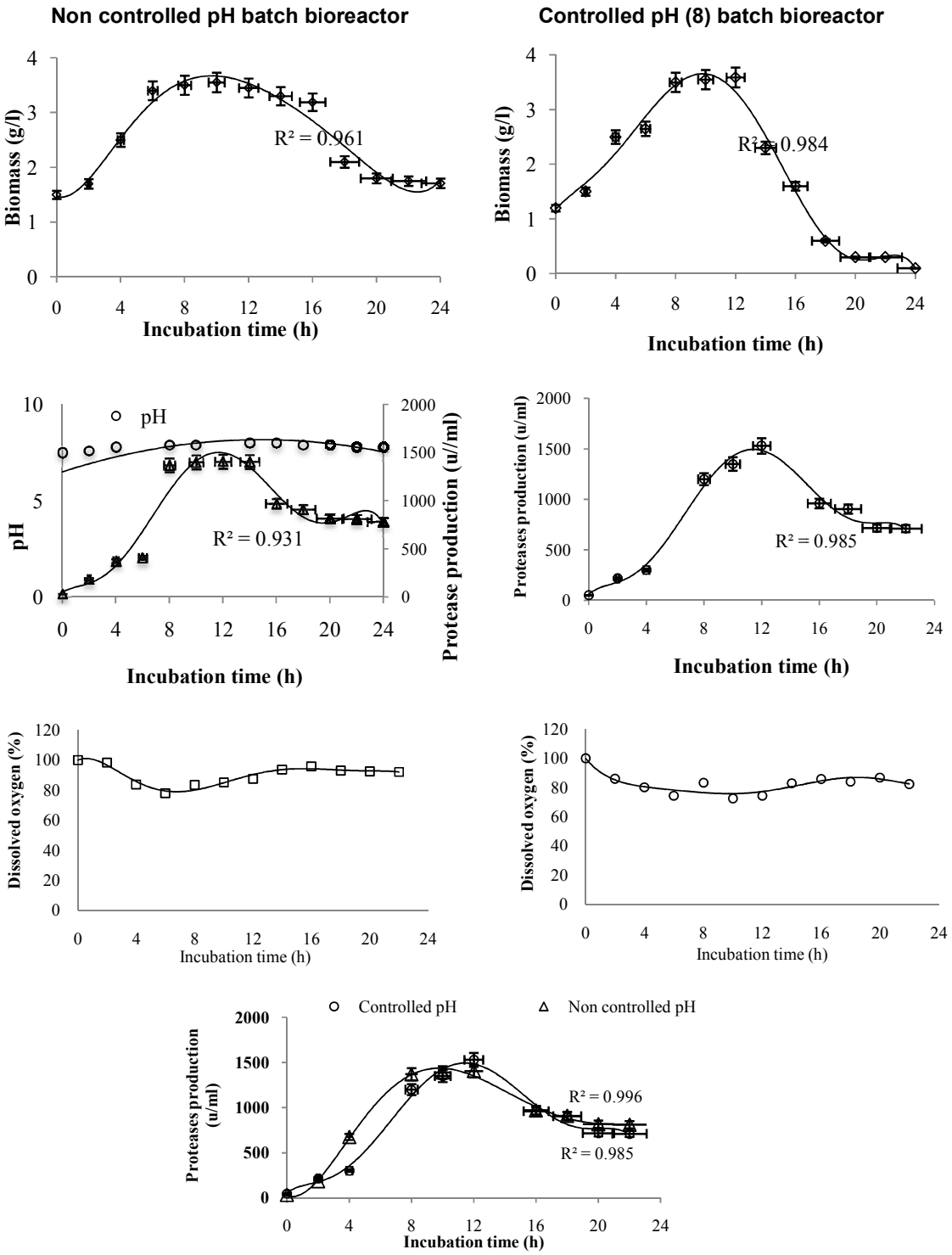


Fig. 3. Cultivation of *B. amyloliquefaciens* 35s in pH-controlled and non-controlled batch culture of modified TGY medium for 24 hours at 37°C and monitoring of biomass, pH change, dissolved oxygen and proteases production. Error bars with 5% value are shown

Proteases produced from the bioreactor experiments were subject to enzymes extraction and purification, characterization and applications of the purified proteases. Results of these later experiments will be published in the next manuscript.

4. CONCLUSION

Instead of conventional method of one variable at time approach, Response Surface Methodology, as statistical approach, showed to be adequate and efficient to optimize protease production by *Bacillus amyloliquefaciens*.

The adequacy of the model was determined, where the "Model F-value" of 106.92 and low probability value < 0.0500 (Prob. >F) implied that the model was significant. Factors, evidencing values of less than 0.05 (Prob. > F) were considered to have significant effects on the response. Among the significant media components, peptone and starch, with confidence level 95%, were further optimized using FCCCD. Conditions promoted proteases production were different from those enhanced cell growth. Physical parameters indicated that production of proteases by *Bacillus amyloliquefaciens* is non-growth dependent.

The validation of optimized conditions, suggested by the model of FCCCD for physical factors was done experimentally. Maximum predicted proteases production (992.12 u/ml) was observed near the mid-point (0) values (concentrations) of both peptone and starch. FCCCD model for nutritional factors was done experimentally to validate the model, and experimental value (935 u/ml) was close to the predicted value (992.12 u/ml) which indicates validation of the model.

Protease production by was scaled-up in 5L bioreactor, in controlled and non-controlled pH batch culture experiments, applying all the optimized conditions determined from the shake flask experiments. In non-controlled pH batch, maximum protease production was 1505u/ml at pH 8 after 12h of incubation. In controlled pH batch experiment, final proteases production in the bioreactor had a yield of 1530 u/ml after 12h incubation at 0.6 vvm aeration and 120 rpm of agitation speed compared to 939 (u/ml) obtained by shake flask cultures after 24 h, which is 1.6 fold over that obtained by the shake flasks experiment.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

REFERENCES

1. Banik RM, Prakash M. Laundry detergent compatibility of the alkaline protease from *Bacillus cereus*. Microbiol Res. 2004;159: 135–140.
2. Beg QK, Sahai V, Gupta R. Statistical media optimization and alkaline protease production from *Bacillus mojavensis*in a bioreactor. Proc Biochem. 2003;39:203–209.
3. Kumar CG, Takagi H. Microbial alkaline proteases: From a bio industrial view point. Biotechnol Adv. 1999;17:561–594.
4. Joo HS, Chang CS. Oxidant and SDS–stable alkaline protease from a halotolerant *Bacillus clausii*l–52: enhanced production and simple purification. J Appl Microbiol. 2006;98:491–497.
5. Myers RH, Montgomery DC. Response surface methodology: Process and product optimization using designed experiments. 1995; New York: John Wiley & Sons, Inc.
6. Box GEP, Wilson KB. On the experimental attainment of optimum conditions. J Royal Statist Soc.1951;13:1–45.
7. Varela H, Ferrari MD, Belobradjic L, Weyrauch R, Loperena ML. Effect of medium composition on the production by a new *Bacillus subtilis*isolate of protease with promising unhairing activity. World J Microbiol Biotechnol. 1996;12:643–645.
8. Chauhan B, Gupta R. Application of statistical experimental design optimization of alkaline protease production from *Bacillus* sp. RGR–14. Proc Biochem. 2004;39:2115–2122.
9. Ramnani P, Kumar SS, Gupta R. Concomitant production and downstream processing of alkaline protease and biosurfactant from *Bacillus licheniformis* RG1: Bioformulation as detergent additive. ProcBiochem. 2005;40:3352–3359.
10. Tari C, Genckal H, Tokath F. Optimization of growth medium using a statistical approach for the production of an alkaline protease from a newly isolated *Bacillus* sp.L21. Proc Biochem. 2006;41:659–665.
11. Reddy LVA, Wee YJ, Ryu HW. Purification and characterization of an organic solvent and detergent–tolerant novel protease

produced by Bacillus sp. RKY3. J Chem Technol Biotechnol. 2008;83:1526–1533.
12. Oskouie SFG, Tabandeh F, Yakhchali B, Eftekhar F. Response surface optimization of medium composition for alkaline protease production by *Bacillus clausii*. J Biochem Eng. 2008;39:37-42.
13. Akolkar A, Bharambe N, Trivedi S, Desai A. Statistical optimization of medium components for extracellular protease production by an extreme haloarchaeon, *Halobacterium* sp. SP1(1). Lett Appl Microbiol. 2009;48:77–83.
14. Zhou LH, Zhang YQ, Wang RJ, Shen XL, Li YQ, Guan WJ. Optimization of mycelial biomass and protease production by *Laccocephalum mylittae* in submerged fermentation. Afr J Biotechnol. 2009;8: 1591–1601.
15. Rai SK, Mukherjee AK. Statistical optimization of production, purification and industrial application of a laundry detergent and organic solvent–stable subtilisin–like serine protease (Alzwiprase) from *Bacillus subtilis* DM–04. BiochemEng J. 2010;48: 173–180.
16. Manikandan M, Pasic L, Kannan V. Purification and biological characterization of a halophilic thermos table protease from *Haloferax lucentensis* VKMM 007. World J Microbiol Biotechnol. 2009;25:2247–2256.
17. Hameed A, Keshavarz T, Evans CS. Effect of dissolved oxygen tension and pH on the production of extracellular protease from a new isolate of *Bacillus subtilis* K2, for use in leather processing. J Chem Technol Biotechnol. 1999;74:5–8.
18. Beshay U, Moreira A. Repeated batch production of alkaline protease using porous sintered glass as carriers. Proc Biochem. 2003;38:1463–1469.
19. Nassar FR, Abdelhafez AA, El-Tayeb TS, Abu-Hussein SH. Proteases production by a bacterial isolate *Bacillus amyloliquefaciens* 35s obtained from soil of the Nile Delta of Egypt. Brit Microbiol Res J; 2015. (accepted for publication).
20. APHA (American Puplic Health Association). Compendium methods of food microbiology 8th ed. APHA, INC. New York; 1992.
21. Sabine L, Everitt BS. A handbook of statistical analyses using SPSS. Boca raton, Fl: Chapman & Hall / CRC. ISBN 1–58488–369–3; 2004.
22. Abu–Sayem SM, Alam MJ, Hoq MM. Effect of temperature, pH and metal ions on the activity and stability of alkaline proteases from novel *Bacillus licheniformis* MZK03. Proc Pakistan Acad Sci. 2006;43:257–262.
23. Lowry OH, Rosebrough NJ, Farr *AL*, Randall RJ. Protein measurement with the Folin phenol reagent. J Biol Chem. 1951;193:265–75.
24. Anson ML. The estimation of pepsin, trypsin, papain and cathepsin with hemoglobin. J. Gen Physiol. 1938;22:79–89.
25. Gamal Rawia F, Nassar Fatma R, Abd El–Hady Hemmat M, El–Sawy M. Glycerol production by osmotolerant yeast strain using fermentor as fed batch and continuous culture techniques. Annal Agric Sci, Ain Shams University, Cairo, Egypt. 1991;36:319–421.
26. Venugopal M, Saramma AV. Characterization of alkaline protease from *Vibrio fluvialis* strain VM 10 isolated from mangrove sediment sample and its application as a laundry detergent additive. Proc Biochem. 2006;41:1239–1243.
27. Genckal H, Tari C. Alkaline protease production from alkalophilic *Bacillus* sp. isolated from natural habitats. Enz Microbial Technol. 2006;39:703–710.
28. Prakasham RS, Rao C, Rao RS, Sarma PN. Alkaline Protease Production By An Isolated *Bacillus Circulans* Under Solid-State Fermentation Using Agro-Industrial Waste: Process Parameters Optimization. Biotechnol Prog. 2005;21: 1380–1388.

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