Journal of Scientific & Industrial Research
Vol. 79, June 2020, pp. 491-498

Statistically Designed Bioprocess for Enhanced Production of Alkaline Protease in *Bacillus cereus* HP_RZ17

H P Jadhav[1], R Z Sayyed[1]*, S S Shaikh[1], H M Bhamre[2], Kumari Sunita[3] and H A El Enshasy[4,5]

[1]Department of Microbiology, P S G V P Mandal's Arts, Science, and Commerce College, Shahada 425 409 (MS), India

[2]S S V P S L K Dr P R Ghogrey Science College, Dhule 424 005 (MS), India

[3]Department Botany, DDU Gorakhpur University, Gorakhpur (UP), India

[4]Institute of Bioproduct Development (IBD), Universiti Teknologi Malaysia (UTM), Johor Bahru, Malaysia

[5]City of Scientific Research and Technology Applications, New Burg Al Arab, Alexandria, Egypt

Received 23 September 2019; revised 22 February 2020; accepted 18 April 2020

Alkaline protease is one of the bulk enzymes having wide commercial demand for various applications. It is commercially produced by a submerged fermentation process employing various bacteria, *Bacillus* sp. being the most widely used species. Statistical optimization of the process for the production of alkaline proteases from rhizospheric bacteria and its application in the biocontrol of plant pathogens has not been explored fully and needs to be studied for the development of efficient bioprocess. We report the enhanced production of alkaline protease in the minimal salt medium (MSM) optimized using statistical approaches such as Plackett Burman Design (PBD) and Response Surface Methodology (RSM). In the first step; PBD, among the total eight variables, three variables namely, yeast extract (p<0.05), fructose (p<0.05) and pH (p<0.05) influenced the production of alkaline protease by *Bacillus cereus* HP_RZ17. These three variables were further analyzed in the second step i.e. Central Composite Design (CCD) of RSM. The optimum yield of alkaline protease by *B. cereus* HP_RZ17 (130.72 UmL^{-1}) was obtained under the optimal conditions such as yeast extract (0.899% w/v), fructose (0.873% w/v), and pH (11.25) of production media. The statistically optimized values of variables used for the scale-up of the process at 5 L capacity bioreactor enhanced the alkaline protease yield (132.48 UmL^{-1}) by 1.09 fold vis-à-vis un-optimized protocol (121.96 UmL^{-1}) in *B. cereus* HP_RZ17.

Keywords: Central Composite Design, Optimization, Plackett Burman Design, Response Surface Methodology, Scale-up

Introduction

Alkaline proteases [EC.3.4.21-24, 99] are commercially versatile enzymes capturing about 45% of the total global enzyme market.[1] Owing to the broad range of working temperature (10–80°C) and pH (4–12)[2–6], microbial protease; especially those produced by *Bacillus* sp. have a huge demand in the enzyme market while protease produced by rhizobacteria have been extensively used in agriculture for the biocontrol of a wide variety of phytopathogens.[7]

Members of the genus *Bacillus* constitute an important group of plant growth promoting rhizobacteria (PGPR).[8,9] Genus *Bacillus* constitutes one of the largest easily cultivable, fastidious groups of PGPR which improve growth and yield of crops.[9] Bacilli, due to their strong rhizosphere colonization, ability to produce very hard spores which survive for prolonged periods in soil[10] and salinity tolerance have been seen as effective bio-inoculants for plant growth promotion.[11] Members of *Bacillus* species successfully colonize the rhizospheric regions[12] and contribute to better growth and yield of crop plants. They have been comprehensively studied due to their effective plant growth promoting ability and antagonistic activity against wide range of phytopathogens.[13] *Bacillus* sp. promote plant growth through mineral nutrition, nitrogen fixation and production of phytohormones and hydrolytic enzymes.[14]

Statistical based optimization offers the best alternative solution to overcome these demerits of OVAT.[15–18] The present research work was focused on the optimization of different variables of production media based on statistical approaches, such as PBD and RSM by CCD to escalate the production of alkaline protease in *B. cereus* HP_RZ17.

Materials and Methods

Source of culture

The isolate *Bacillus cereus* HP_RZ17 used in the present study was obtained from culture repository of

*Author for Correspondence
E-mail: sayyedrz@gmail.com

Department of Microbiology of PSGVPM's ASC College, Shahada, India.[19]

Evaluation of medium variables by OVAT for the production of alkaline protease

The influence of various physicochemical factors like incubation period (0–96 h) and temperature (20–50°C), media pH (4.00–12.00), inoculum concentration (0.5–2.0% v/v), nitrogen (1.0% w/v) (casein, tryptone, yeast extract, urea, ammonium nitrate (NH_4NO_3) and ammonium chloride (NH_4Cl_2) and carbon sources (1.0% w/v) (glucose, fructose, sucrose, lactose, maltose, and dextrose) on the production of alkaline protease in *B. cereus* HP_RZ17 were initially studied individually while keeping the other constituents constant using the OVAT methodology. The alkaline protease production was carried out in MSM containing (gL^{-1}), KH_2PO_4; 0.002, $MgSO_4$; 0.005, NaCl; 0.005, $CaCl_2$, 0.005, yeast extract; 10, fructose; 10, pH 11.[20,21] The lag phase batch culture of *B. cereus* HP_RZ17 (1.5% v/v) was grown in MSM at 30°C in shaking at 120 rpm for 48 h.[22,23] Following the incubation, the broth was centrifuged at 10,000 rpm for 15 min at 30°C and the resulting supernatant was subjected to protease assay.[19]

Protease assay

Protease activity was estimated in terms of proteolysis of a substrate (casein) and the release of tyrosine units. The cell-free supernatant (crude enzyme) and 0.65% (w/v) casein were incubated in 20 mM phosphate buffer pH 7.0 for 10 min at 37°C, followed by the addition of 110 mM Tri-chloroacetic acid (TCA) to stop the enzyme reaction. Cell-free supernatant was obtained following the centrifugation (10,000 rpm for 10 min) of precipitated protein, was subjected for the protease assay and the number of tyrosine units produced due to proteolysis were estimated.[24] The total protein content was measured as per the method of Lowry *et al*.[25] using Bovine Serum Albumin (BSA) as a standard. One unit of alkaline protease activity was defined as the amount of the enzyme that liberated 1 µg tyrosine mL^{-1} min^{-1} from casein under specified assay conditions.[19,26]

Statistical-based optimization of production medium

Identification of significant media components that influence the production of alkaline protease is the most crucial step in the optimization of parameters.[27,28] The significant variable(s) were optimized in the first step i.e. PBD while in second step RSM (through CCD) concentration of significant variable(s) was analyzed for the optimum production of alkaline protease by *B. cereus* HP_RZ17.[29,30]

Evaluation of significant variable using PBD

To investigate the significant variables for the optimum production of alkaline protease, eight variables were selected in PBD. This design was based on the two-factorial or multi-factorial experiments, which facilitate to ascertain that the variables have a significant role in the production of an enzyme.[31] The physicochemical parameters that are most likely to affect alkaline protease production preferred were K_2HPO_4 (A), $MgSo_4{\cdot}7H_2O$ (B), $CaCl_2$ (C), NaCl (D), yeast extract (E), fructose (F), inoculum concentration (G) and pH (H). Each independent variable was examined at two different levels i.e. low (-1) and high (+1).

The PBD experimental design comprised of 12 sets of different levels of eight variables. The effects of all variables on alkaline protease production were calculated and analyzed by one-way Analysis of Variance (ANOVA). Based on the *p*-value ($p<0.05$) and the highest confidence level <95% of significant variables were selected from other independent variables (Table 1). The magnitude of the selected eight variables was estimated using the Pareto chart analysis (Fig. 1).[32,33]

Table 1 — Experimental PBD data analysis using ANOVA

Component code	Components	Effect	coefficient	*t* - value	*p*-value
Constant			105.95	101.17	0
A	K_2HPO_4 (%)	-4.30	-2.15	-2.05	0.132
B	$MgSO_4$ (%)	-4.22	-2.11	-2.01	0.137
C	$CaCl_2$ (%)	-1.21	-0.60	-0.58	0.605
D	NaCl (%)	-5.32	-2.66	-2.54	0.085
E	Yeast extract (%)	19.80	9.90	9.46	<0.003
F	Fructose (%)	12.61	6.30	6.02	<0.009
G	Inoculum (%)	4.71	2.36	2.25	0.110
H	pH	20.37	10.19	9.73	<0.002

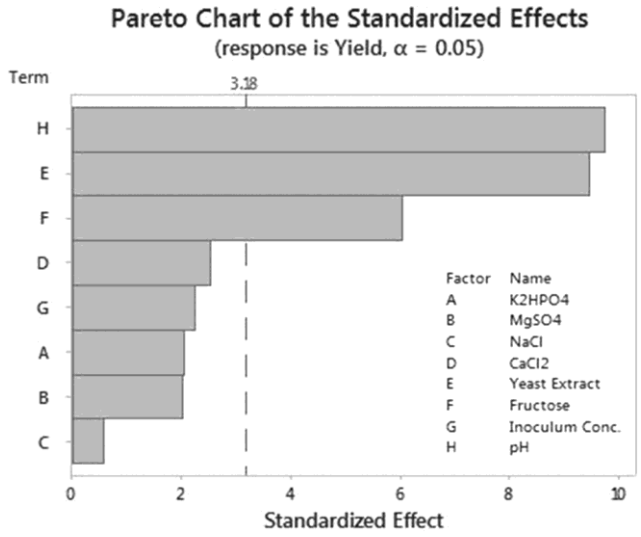

Fig. 1 — Pareto chart representing the effects of medium variables on alkaline protease production by *B. cereus* HP_RZ17

Optimization of significant medium variables by CCD of RSM

The significant variables of production medium obtained from PBD were analyzed to find the optimum region, type of response by variable in that and the data validation using CCD[34] of RSM.[18] The optimized values of variables were illustrated by considering the fractional factorial, distance α from its center and a central point. In CCD the interactions between significant variables were studied by which the optimal level of the variable can be decided. Among the eight variables identified in PBD yeast extract (E), fructose (F) and pH (H) were identified as significant variables. Hence these variables were picked up for further optimization at five different levels i.e. -α, -1, 0, +1 and +α. The range of extremely low (α) to extreme high (+α) values was considered in CCD. The twenty different experimental sets comprising of eight factorial points, six axial points and central points (Table 2) were experimentally performed and the effects of independent variable or response were calculated in the form of yield (Y) and were expressed as UmL^{-1}.

The experimental results of RSM were subjected to ANOVA and fitted into the following second-order polynomial equation (Eq. 1). It generated an empirical model that relates to the responses obtained in the independent variable to the experiment. A second-order polynomial equation (Eq. 1) was then fitted to the response by multiple regression procedures. This resulted in an empirical model that related the response measured in the independent variables to the experiment.

Table 2 — Experimental data obtained for significant variables obtained from PBD in CCD

Run	Type	E	F	H	Enzyme yield (UmL^{-1})	
					Experimental	Predicted
1	Factorial	-1	-1	-1	90.52	90.45
2	Factorial	1	-1	-1	100.27	106.38
3	Factorial	-1	1	-1	83.64	86.77
4	Factorial	1	1	-1	109.56	114.51
5	Factorial	-1	-1	1	96.75	94.55
6	Factorial	1	-1	1	99.86	99.48
7	Factorial	-1	1	1	112.67	109.31
8	Factorial	1	1	1	123.24	126.06
9	Axial	-1.6818	0	0	90.55	93.36
10	Axial	1.68179	0	0	127.54	120.84
11	Axial	0	-1.6818	0	95.61	94.88
12	Axial	0	1.68179	0	117.29	114.13
13	Axial	0	0	-1.6818	103.57	96.50
14	Axial	0	0	1.68179	106.48	109.66
15	Central	0	0	0	128.97	128.93
16	Central	0	0	0	128.73	128.93
17	Central	0	0	0	128.24	128.93
18	Central	0	0	0	128.45	128.93
19	Central	0	0	0	129.34	128.93
20	Central	0	0	0	129.18	128.93

$$Yi = \beta_0 + \sum \beta_i X_i + \sum \beta_{ii} X_i^2 + \sum \beta_{ij} X_i X_j \quad \ldots (1)$$

Where

Yi – predicted response,

X_iX_j - input variables (influence the response variable Y),

β_0 - constant,

β_i - i^{th} linear coefficient,

β_{ii} - quadratic coefficient,

β_{ij} - ij^{th} interaction coefficient.

The experimental run with the optimized level of the significant medium variables was performed to validate the predicted model.

Scale-up at laboratory scale (5L) bioreactor

For the validation of the process designed at shake flask level production of alkaline protease by *B. cereus* HP_RZ17, the protocol was scaled-up to 5L capacity on a fully-automated laboratory bioreactor [Model LF-5 Murhopye Scientific Co., Mysore, India].

Software and data analysis

Statistical and graphical analysis of experimental data of PBD and RSM was performed with the software Minitab 18 (Minitab GmbH, Munich, Germany).

Results and Discussion

Evaluation of medium variables by OVAT for the production of alkaline protease

The effective levels of physicochemical i.e. incubation period 48 h, incubation temperature 30°C, media pH 11.0, inoculum concentration 1.5%, carbon source (fructose) 1% and nitrogen source (yeast extract) 1% on optimum alkaline protease production were studied by OVAT methodology[19], the optimum production of an enzyme in MSM by *B. cereus* HP_RZ17 was achieved up to 121.96 UmL^{-1}. Chandra *et al.*[35] reported the influence of various media parameters on protease production by *Bacillus* sp. (GU566359) evaluated using the OVAT approach where they achieved 470 UmL^{-1} of enzyme production. Polley and Ghosh[36] reported the influence of media components on alkaline protease yield in *A. alternata* TUSGF1 and have claimed an optimum enzyme yield of 96 UmL^{-1}.

Evaluation of significant variable using PBD

The limitation of OVAT to exploit the effect of interaction between media components becomes the major factor to obtain maximum yield in scale-up. To overcome this issue the PBD and RSM have been found as the best advances in the field of media optimization.[37] Using the PBD the most significant and influential variables were identified for the maximum alkaline protease production from *B. cereus* HP_RZ17. The standardized effects of eight independent variables analyzed by ANOVA are graphically represented in the Pareto chart (Fig. 1). The yield of alkaline protease produced from *B. cereus* HP_RZ17 in 12 sets of the experiment proposed by PBD estimated under standard conditions was observed in the range of 87.59 – 130.65 UmL^{-1}. Analysis of regression coefficients and *t* -the value of eight variables of the proposed PBD showed that among the tested variables yeast extract (E) (p=0.003), fructose (F) (p=0.009) and pH (H) (p=0.002) positive effects on alkaline protease production. These three variables were found significant for alkaline protease production at a 95% confidence level (α= 0.05) (Fig. 1) and hence these variables were chosen for further optimization by RSM.

Optimization of significant medium variables by CCD of RSM

In the initial screening of variables by PBD, three variables namely E, F, and H came out as significant variables for the production of alkaline protease by *B. cereus* HP_RZ17. The experimental results of CCD for alkaline protease production showed a good fit with the predicted values (Table 2).

In RSM the variables of Eq. (1) were evaluated using multiple regression analysis. The second-order polynomial regression equation showed the empirical relationship between the alkaline protease yield (Y) and the significant variables of PBD in coded units, the yield was calculated by Eq. (2).

$$Y = -1121 + 303.4\,(E) - 8.2\,(F) + 199.4\,(H) - 123.5\,(E^2) - 138.2\,(F^2) - 9.14\,(H^2) + 47.3\,(E*F) - 10.99\,(E*H) + 18.45\,(F*H) \quad \ldots (2)$$

The ANOVA of the second-order response surface model evaluated by Fisher's statistical F-test showed that the regression model is highly significant for the production of alkaline protease by *B. cereus* HP_RZ17. The F-value of square regression was higher vis-à-vis the tabulated p<F (Table 3); The results of variables, interaction in different forms that effects of linear and square interaction, the square interaction between among the model terms of E, F, and H variables were significant, while in 2-way interaction the effect of interaction between F and H were found to be non-significant (Fig. 2). By evaluating the coefficient of determination (R^2) and adjusted R^2 the goodness-of-fit of the model was examined. The higher R^2 represented the maximum variation in observations of the fitted regression equation model.[38,39] The resulting high R^2 value reflected that the fitted model could explain 95.32% of

Table 3 — ANOVA of a quadratic model for alkaline protease production from *B. cereus* HP_RZ17

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Model	9	4489.02	498.78	22.65	<0.000
E: Yeast Extract	1	911.30	911.30	41.39	<0.000
F: Fructose	1	447.45	447.45	20.32	<0.001
H: pH	1	208.99	208.99	9.49	<0.012
E^2	1	858.33	858.33	38.98	<0.000
F^2	1	1074.54	1074.54	48.81	<0.000
H^2	1	1203.59	1203.59	54.67	<0.000
EF	1	69.80	69.80	3.17	>0.105
EH	1	60.45	60.45	2.75	>0.129
FH	1	170.11	170.11	7.73	<0.019
Error	10	220.17	22.02	—	—
Lack-of-Fit	5	219.27	43.85	242.58	<0.000
Pure Error	5	0.90	0.18	—	—
Total	19	4709.19	—	—	—

R^2=95.32%, R^2(pred.) = 64.32%, R^2(Adj.)= 91.12%, (p<0.05 Significant)

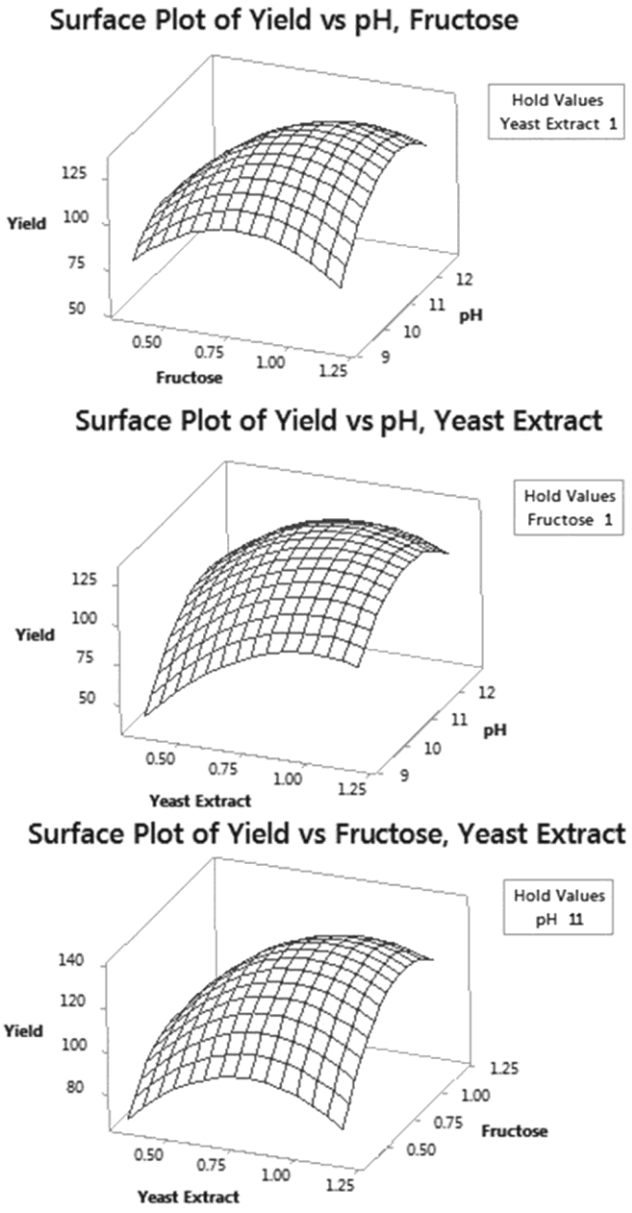

Fig. 2 — The 3D surface plots representing the response of interaction between carbon source - media pH, nitrogen source - media pH and carbon source - nitrogen source on alkaline protease production from *B. cereus* HP_RZ17

the total variation. The adjusted R^2 corrects the R^2 value for the sample size and the number of terms in the model. The adjusted R^2 value (91.12%) in the present study supported the high significance of the model. The standard error of the regression (*S*) of 4.32 which represents that the observations of model fall in the regression line and show the good relation between predicted and experimental response. These results state that the model provided for the CCD experiment by the response equation are appropriate.[18,39]

The 3D-surface plots illustrated the optimum concentrations of each significant variable while interacting with other media variables for the maximum production. The 2D-counter plots of the relationship between fitted responses of two continuous significant variables were observed by contour lines of the points that have the same response value in production. The 3D-surface plots (Fig. 2) and 2D-counter plots (Fig. 3) displayed the various effective regions along with the highest yielding point of the alkaline protease by each variable.

The pH is one of the important physical variables as it controls various enzymatic processes and molecular transportation through the cell membrane. Changes in media pH disturb the equilibrium of the system by fluctuating the H^+ and OH^- concentration and media nutrients. The optimum pH for most of the alkaliphilic *Bacillus* strains has been recorded in the range 9.0–11.0.[40] The alkaline protease production is strongly influenced by the various media constituents such as carbon and nitrogen sources. The growth rate and enzyme production rely on the nutritional requirement of strain producing alkaline protease and the nature or type of carbon and nitrogen source may vary as per the requirement of alkaline protease producing organism. The complex medium composed of optimum concentrations of these nutrient sources was found to be the best for the optimum production of alkaline protease.[41] Patel *et al*.[42] and Solangi *et al*.[43] reported the optimum alkaline protease production by *B. cereus AG1* and *B. subtilis* in a statistically optimized complex medium containing fructose and yeast extract respectively.

Using the CCD experimental results, Minitab18 (software) predicted the optimum concentrations of yeast extract (E= 0.899%), fructose (F=0.873) and pH (H= 11.25) and their response as alkaline protease yield (133.21 UmL^{-1}). The predicted model was analyzed experimentally in triplicate, and the results were as per the predicted yield of alkaline protease (130.72 UmL^{-1}) which validates the quadratic model predicted by Minitab at shake flask level.

Scale-up at laboratory scale (5L) bioreactor level

Statistically optimized concentrations of the significant variables when used for scale-up from shake flask level to 5L lab bioreactor, a significant increase in the yield of alkaline protease was evident from 130.72 UmL^{-1} to 132.84 UmL^{-1}. A 1.01 (statistically shake flask to bioreactor) fold or 1.08

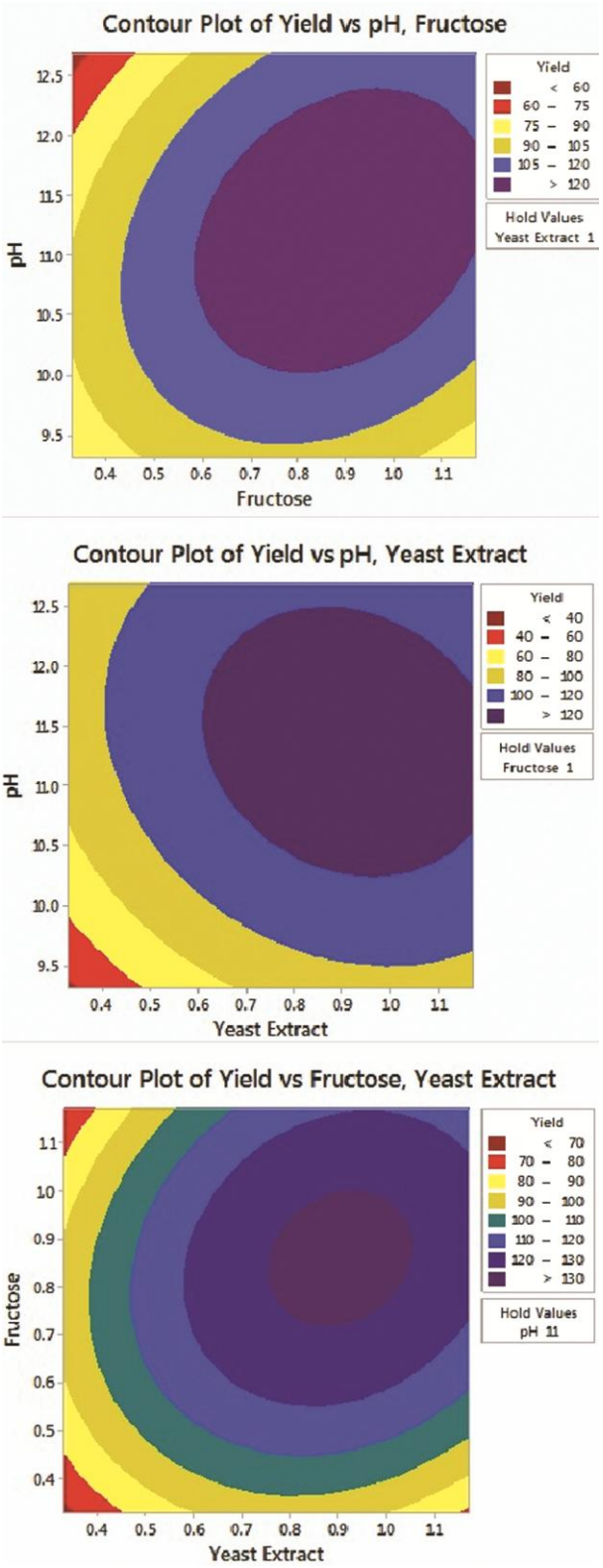

Fig. 3 — Counter plots showing the combined effects of carbon source - media pH, nitrogen source - media pH and carbon-nitrogen source on alkaline protease production from *B. cereus* HP_RZ17

(un-optimized 121.96 UmL^{-1} to statistically optimized bioreactor) fold increase in the yield of alkaline protease was obtained due to statistical optimization. Limkar *et al*.[30] reported 1.33 fold increases in the alkaline protease production in *Bacillus* sp. using a statistical optimization approach.

Conclusions

The statistical approach facilitates the combinations of experiments to elucidate the significant variables of the production medium for optimum production of alkaline protease in *B. cereus* HP_RZ17. The significant variables initially screened by PBD were further optimized by using RSM. The successfully scale up of statistical-based shake flask process to laboratory scale (5L) bioreactor validated the variables and their concentrations studied at the shake flask level. The values of variables namely yeast extract (E), fructose (F) and pH (H) predicted at shake flask level further enhanced the enzyme yield by 1.09 fold (from 121.96 to 132.84 UmL^{-1}). This reflects the usefulness of the application of statistical optimization in enzyme production. The experimental results obtained showed a good relationship with the values predicted by Minitab 18 (software). Thus present results illustrated that the statistically optimized bioprocess could be the best suitable approach for enhancing the productivity of alkaline protease at an industrial scale.

References

1 Chew L Y, Toh G T & Ismail A, Application of proteases for the production of bioactive peptides, in *Enzymes in Food Biotechnology*, edited by Kuddus M (Academic Press, London) 2019, 247–261. https://doi.org/10.1016/B978-0-12-813280-7.00015-3

2 El Enshasy H, Abuoul-Enein A, Helmy S & El Azaly Y, Optimization of the industrial production of alkaline protease by *Bacillus licheniformis* in different production scales, *Aust J Basic Appl Sci*, **2** (2008) 583–593.

3 Sumantha A, Larroche C & Pandey A, microbiology and industrial biotechnology of food-grade proteases: a perspective, *Food Technol Biotechnol*, **4** (2006) 211–220.

4 Homaei A, Lavajoo F & Sariri R, Development of marine biotechnology as a resource for novel proteases and their role in modern biotechnology, *Int J Biol Macromol*, **88** (2016) 542–552. https://doi.org/10.1016/j.ijbiomac.2016.04.023

5 Jadhav H P & Sayyed R Z, Hydrolytic enzymes of rhizospheric microbes in crop protection, *MOJ Cell Sci Rep*, **3** (2016) 135–136. https://doi.org/10.15406/mojcsr.2016.03.00070

6 Sayyed R Z, Ilyas N, Tabassum B, Hashem A, Abd_Allah E F & Jadhav H P, Plausible Role of Plant Growth-Promoting Rhizobacteria in Future Climatic Scenario, in *Environmental*

Biotechnology: For Sustainable Future, edited by Ranbir C S, Naveen K A, Richa K (Springer, Singapore) 2019, 175–197. https://doi.org/10.1007/978-981-10-7284-0_7

7 Jadhav H P, Shaikh S S & Sayyed R Z, Role of Hydrolytic Enzymes of Rhizoflora in Biocontrol of Fungal Phytopathogens: An Overview, in *Rhizotrophs: Plant Growth Promotion to Bioremediation*, edited by Samina M, (Springer, Singapore) 2017, 183–203. https://doi.org/10.1007/978-981-10-4862-3_9

8 Parray J A, Jan S, Kamili A N, Qadri R A, Egamberdieva D & Ahmad P, Current perspectives on plant growth-promoting rhizobacteria, *J Plant Growth Regul*, **35** (2016) 877–902.

9 Kalam S, Das S N, Basu A & Podile A R, Population densities of indigenous *Acidobacteria* change in the presence of plant growth promoting rhizobacteria (PGPR) in rhizosphere, *J Basic Microbiol*, **57(5)** (2017) 375–386, 10.1002/jobm.201600588.

10 Nelson W H, Dasari R, Feld M & Sperry J F, Intensities of calcium dipicolinate and *Bacillus subtilis* spore Raman spectra excited with 244 nm light, *Appl Spectrosc*, **58** (2004) 1408–1412.

11 Vurukonda S S K P, Vardharajula S, Shrivastava M & SkZ A, Enhancement of drought stress tolerance in crops by plant growth promoting rhizobacteria, *Microbiol Res*, **184** (2016) 13–24.

12 Zhou D, Huang X F, Chaparro J M, Badri D V, Manter D K & Vivanco J M, Root and bacterial secretions regulate the interaction between plants and PGPR leading to distinct plant growth promotion effects, *Plant Soil*, **401** (2016) 259–272.

13 Ashwini N & Srividya S, Potentiality of *Bacillus subtilis* as biocontrol agent for management of anthracnose disease of chilli caused by *Colletotrichum gloeosporioides* OGC1, *Biotech*, **4** (2014) 127–136.

14 Barea J M & Richardson A E, Phosphate mobilisation by soil microorganisms, in *Principles of plant-microbe interactions*, edited by Lugtenberg (Springer International Publishing, Heidelberg, Switzerland) 2015, 225–234.

15 Abdel-Fattah YR, El Enshasy H, Anwar M, Omar H, Abou El magd E & Abou Zahra R, Application of factorial experimental designs for optimization of Cyclosporin A production by *Tolypocladium inflatum* in submerged culture, *J Microbiol Biotechnol* **17** (2007) 1930–1936.

16 Abdelgalil S A, Attia A M, Reyed R M, Soliman N A & El Enshasy H, Application of experimental design for optimization the production of *Alcaligenes faecalis* NYSO laccase, *J Sci Appl Res* **77** (2018) 713–722.

17 Shaikh S S, Wani S J & Sayyed R Z, Statistical-based optimization and scale-up of siderophore production process on laboratory bioreactor, *3 Biotech*, **6** (2016) 69. https://doi.org/10.1007/s13205-016-0365-2

18 Bhamare H M, Jadhav H P & Sayyed R Z, Statistical optimization for enhanced production of extracellular laccase from *Aspergillus* sp. HB_RZ4 isolated from bark scrapping, *Environ Sustain*, **1** (2018) 159–166. https://doi.org/10.1007/s42398-018-0015-1

19 Jadhav H P, Sonawane M S, Khairnar M H & Sayyed R Z, Production of hydrolytic enzymes by *Bacillus cereus* HP_RZ17 and *Paneibacillus* sp. HP_RZ19, *Environ Sustain*, (2020) 1–9. https://doi.org/10.1007/s42398-020-00096-z

20 Santhi R, Microbial production of protease by *Bacillus cereus* using cassava waste water, *Eur J Exp Biol*, **4** (2014) 19–24.

21 Ash K & Ramteke P W, Optimization of extracellular alkaline protease production from *Pseudomonas aeruginosa* isolated from soil samples, *Int J Agri Environ Biotechnol*, **11** (2018) 187–194. https://doi.org/10.30954/0974-1712.2018.00178.24

22 Abdel Fattah Y R, El Enshasy H A, Soliman N A & El-Gendi H, Bioprocess Development for the production of alkaline protease by *Bacillus psudoformus* Mn6 through statistical experimental designs, *J Microbiol Biotechnol* **19** (2009) 378–386.

23 Tambekar S D & Tambekar D H, Optimization of the production and partial characterization of an extracellular alkaline protease from thermo-halo-alkalophilic lonar lake bacteria, *Biosci Discov*, **4** (2013) 30–38.

24 Cupp-Enyard C, Sigma's Non-specific Protease Activity Assay - Casein as a Substrate, *J Vis Exp*, **19** (2008) e899.

25 Lowry O H, Rosebrough N J, Farr A L & Randall R J, Protein measurement with the Folin phenol reagent, *J Biol Chem*, **193** (1951) 265–275.

26 Bouacem K, Bouanane-Darenfed A, Laribi-Habchi H, Elhoul M B, Hmida-Sayari A, Hacene H, Ollivier B, Fardeau M L, Jaouadi B & Bejar S, Biochemical characterization of a detergent-stable serine alkaline protease from *Caldicoprobacter guelmensis*, *Int J Bio Macromol*, **81** (2015) 299–307. https://doi.org/10.1016/j.ijbiomac.2015.08.011

27 Sharmila G, Nidhi B & Muthukumaran C, Sequential statistical optimization of red pigment production by *Monascus purpureus* (MTCC 369) using potato powder, *Ind Crop Prod*, **44** (2013) 158–64. https://doi.org/10.1016/j.indcrop.2012.11.007

28 Sukumaran S, Dailin D J, Malek R A, Peng T, Hanapi S Z & EL Enshasy H, Production of High Cell Mass of *Bacillus firmus* Using Statistical Medium Optimization, *J Sci Ind Res*, **78** (2019) 358–363. http://nopr.niscair.res.in/handle/123456789/47559

29 Bas D & Boyacı I H, Modeling and optimization I: Usability of response surface methodology. *J Food Eng*, **78** (2007) 836–845. https://doi.org/10.1016/j.jfoodeng.2005.11.024

30 Limkar M B, Pawar S V & Rathod V K, Statistical optimization of xylanase and alkaline protease co-production by *Bacillus* spp using Box-Behnken Design under submerged fermentation using wheat bran as a substrate, *Biocat Agric Biotechnol*, **17** (2019) 455–464. https://doi.org/10.1016/j.bcab.2018.12.008

31 Soltani M, Abd Malek R, Ware I, Ramli S, Elsayed E A, Aziz R & El-Enshasy H A, Optimization of cordycepin extraction from *Cordyceps militaris* fermentation broth, *J Sci Ind Res*, **76** (2017) 355–361.

32 Moorthy I M & Baskar R, Statistical modeling and optimization of alkaline protease production from a newly isolated alkalophilic *Bacillus* species BGS using response surface methodology and genetic algorithm, *Prep Biochem Biotechnol*, **43** (2013) 293–314. https://doi.org/10.1080/10826068.2012.719850

33 Then C, Wai O K, Elsayed E A, Wan Mustapha W A, Othman N Z, Aziz R, Wadaan M & El Enshasy H A, Comparison between classical and statistical medium

optimization approaches for high cell mass production of *Azotobacter vinelandii*, *J Sci Ind Res*, **75** (2016) 231–238.

34 Bijesh K & Sebastian D, Response surface methodological approach to optimize the critical medium components for augmented pectinase production by *Bacillus subtilis* BKDS1, *J Pure Appl Microbiol*, **12** (2018) 981–991. https://doi.org/10.22207/JPAM.12.2.62

35 Chandra S J, Shanker A S & Pindi P K, Isolation and characterization of novel and efficient protease producing bacteria from drinking water resources, *Water Supply*, **20(1)** (2020) 157–164. https://doi.org/10.2166/ws.2019.150

36 Polley T & Ghosh U, Optimization of Process Parameters for Production of Alkaline Protease by OVAT Method Using Isolated Strain *Alternaria alternata* TUSGF1, *Asian Food Sci J*, **9** (2019) 1–8. https://doi.org/10.9734/afsj/2019/v9i430017

37 Unuofin J O, Okoh A I & Nwodo U U, Utilization of agroindustrial wastes for the production of laccase by *Achromobacter xylosoxidans* HWN16 and *Bordetella bronchiseptica* HSO16, *J Environ Manag*, **231** (2019) 222–231.

38 Mukherjee A K, Borah M & Rai S K, To study the influence of different components of fermentable substrates on induction of extracellular α-amylase synthesis by *Bacillus subtilis* DM-03 in solid-state fermentation and exploration of feasibility for inclusion of α-amylase in laundry detergent formulations, *Biochem Eng J*, **43** (2009) 149–156. https://doi.org/10.1016/j.bej.2008.09.011

39 Rai S K & Mukherjee A K, Statistical optimization of product*ion, purification* and industrial application of laundry detergent and organic solvent-stable subtilisin-like serine protease (Alzwiprase) from *Bacillus subtil**is*** DM-04, *Biochem Eng J*, **48** (2010) 173–180. https://doi.org/10.1016/j.bej.2009.09.007

40 Chandra S J, Pallavi P, Anuradha B S & Reddy S R, Optimization of bioprocess for enhanced production of alkaline protease by a *Bacillus subtilis* SHmIIIa through Plackett-Burman design, *Afr J Microbiol Res*, **9** (2015) 1738–1744.

41 Gnanadoss J J & Devi S K, Optimization of nutritional and culture conditions for improved protease production by *Aspergillus nidulans* and *Aspergillus flavus*, *J Microbiol Biotechnol Food Sci*, (2019) 518–523.

42 Patel Y, Gupte A & Gupte S, Production, Partial Purification, Characterization and detergent compatibility of alkaline protease from soil isolate *Bacillus cereus* AG1, *Int J Curr Microbiol App Sci*, **7** (2018) 587–600.

43 Solangi B A, Nawaz H R, Nadeem U, Zehra B & Noor A A, Optimization of Production Condition of Alkaline Protease Using Indigenous *Bacillus subtilis* from Agricultural Soil, *J Biol Sci*, **61** (2018) 84–88.