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Optimization of Alkaline Protease Production by Alkaliphilic *Bacillus* sp. KW2 in Low Cost Medium using Statistical Approaches

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Abstract

Objective: Isolation of alkaline protease producing alkaliphilic bacteria and optimization of protease production in low cost medium.

Methods: Optimization of protease production was performed by one-variable-at-a-time (OVAAT) approach and statistical approaches using Design Expert 6 software.

Results: Nine (9) morphologically distinct alkaliphilic bacterial strains were isolated. Of these isolates 6 strains were found to be positive for protease production. Among these proteolytic strains, *Bacillus* sp. KW2 was selected for protease production optimization studies. The optimal protease production was observed at 30°C and pH 10.7. Among the various carbon and nitrogen sources studied rice bran and soybean meal were found to be the best Carbon and Nitrogen sources respectively. Plackett-Burman design (PBD) was used to screen signal factors that influenced the protease production. Rice bran, soybean meal, inoculum age, $CaCl_2$ and inoculum size gave positive effects whereas KH_2PO_4 and $MgSO_4$ gave negative effects on protease production. The four most critical factors viz. rice bran, soybean meal, $CaCl_2$ and KH_2PO_4 were selected for optimization studies by Response Surface Methodology (RSM). The response surface graphs showed significant interactions among the factors and a final optimized medium (FOM) was obtained after PBD and RSM experiments. Overall, a 4.8 fold increase in protease production was observed after optimization. Studies of the time course of protease production by *Bacillus* sp. KW2 revealed that maximal protease production occurred at the stationary phase (84 h) of growth.

Conclusions: The present study shows that protease production is greatly influenced by cultural conditions and media constituents. This study also established that the strain KW2 could produce alkaline protease in a low cost medium containing rice bran and soybean meal as major carbon and nitrogen sources. KW2 and its protease may be promising agents for biotechnological applications.

Keywords: Alkaline protease; Protease production; Plackett-Burman design (PBD); Response surface methodology (RSM)

Introduction

Alkaline proteases are robust enzymes with considerable industrial applications in detergent, leather processing, pharmaceutical, food processing, feed and chemical industries, as well as in silver recovery and waste treatment [1,2]. The harsh conditions required for industrial applications and the high cost of alkaline protease production warrants continued search for microbial sources of alkaline proteases. The cost of enzyme is a major obstacle in the successful application of proteases in industry. Protease yield has been improved by screening for hyperproducing strains and/or by optimization of the fermentation medium. Strain improvement by either conventional mutagenesis or recombinant DNA technology is one approach for improving the production of proteases [3]. Approximately, 40% of the production cost of industrially important enzymes is estimated to derive from the cost of the growth medium [4,5]. Hence, optimization of fermentation conditions is necessary for cost-effective enzyme production. Media components exert great influence on extracellular protease production and the effects vary in different organisms. Therefore, medium components and their concentrations have to be optimized accordingly [6]. The 'one-variable-at-a-time approach' (OVAAT) of improving fermentation conditions is the most frequently used operation in biotechnology to obtain maximum cell growth and high yields for the desired metabolic product. This approach is not only time consuming but it also ignores the combined interactions among physicochemical parameters [7]. Optimization by statistical approaches such as Plackett-Burman Design (PBD) and Response Surface Methodology (RSM) are generally preferred over OVAAT approach for achieving significant improvements in yield and reduction in the production cost [8]. RSM is a widely accepted statistical approach for modelling and analysis of problems in which a response e.g. level of extracellular enzyme production is influenced by several variables such as temperature, pH and media components. Moreover, several low cost agro-industrial by-products such as rice bran [9], corn steep liquor [10], soybean meal [11], feather meal [12], potato peel [13] and green gram husk [14] have been used as media components to reduce the cost of enzyme production. The present study aims to improve the protease production by *Bacillus* sp. KW2, a strain isolated from waste

dumping sites, in a low cost medium through optimization of medium components and their concentrations using statistical approaches.

Material and Methods

Isolation of alkaliphilic bacteria

Soil samples collected from waste dumping sites at Langol, Manipur, India were serially diluted (10^{-2} to 10^{-7}) in sterile saline solution. The diluted samples were spread plated on Horikoshi medium I [15] and plates were incubated at 30°C for 72 h. Morphologically distinct strains were subcultured and screened for protease production on Horikoshi medium I supplemented with 1% (w/v) skim milk. The pure cultures were preserved as slants at 4°C and as glycerol suspensions (20%, v/v) at -20°C.

Strain selection and Inoculum preparation

The most potent proteolytic strain was selected for further studies. A loopful of bacterial culture was inoculated in Horikoshi-I broth and kept incubated under shaking conditions (30°C, 150 rpm, 24 h). The culture broth was centrifuged (10,000 rpm, 30 min). The pellet was collected, washed twice with sterile distilled water (SDW) and then centrifuged. The pellet was then dissolved in 10 ml SDW and the optical density (OD) was measured at 600 nm. The OD was then adjusted to 0.5.

Enzyme production

Protease production by *Bacillus* sp. KW2 was done in submerged fermentation in glucose yeast extract medium (GYM) containing glucose (1% w/v), yeast extract (0.5%, w/v), $MgSO_4$ (0.05%, w/v), and KH_2PO_4 (0.5 %, w/v). Na_2CO_3 (0.5%, w/v) was autoclaved separately and added to the media. 2% (v/v) inoculum was added in 50 ml production medium and incubated under shaking conditions (30°C, pH 10.5, 150 rpm, 72 h). The culture broth was centrifuged (8,000 rpm, 15 min) and the supernatant was used for protease assay.

Protease assay

Protease activity was determined by using Hammerstein casein as substrate. 1 g casein was dissolved in 100 ml Glycine-NaOH buffer (50 mM, pH 10). 1 ml substrate was added to 1 ml appropriately diluted crude enzyme and incubated at 50°C in a water bath for 10 min. The reaction was stopped by adding 2 ml TCA (10%, w/v) and the reaction mixture was centrifuged (8,000 rpm, 15 min). A control sample (blank) was also included in which TCA was added before the addition of enzyme. Absorbance was measured at 280 nm against blank. One unit of enzyme activity was defined as the amount of enzyme that led to an increase in absorbance of 0.01 at 280 nm per min.

Experimental design for optimization of medium components

Medium optimization was carried out by OVAAT and statistical approaches. Various physiological and nutritional parameters of the production medium were first standardized in GYM medium by OVAAT approach. Following this step, signal parameters affecting protease production were identified by PBD and interactions among signal parameters were studied by RSM.

One-variable-at-a-time approach (OVAAT)

To evaluate the influence of temperature on protease production, fermentation was carried out at various temperatures (25°C, 28°C, 30°C, 32°C, 35°C, 37°C, 40°C and 45°C).

Effects of sodium carbonate on protease production were studied by varying sodium carbonate concentration in the GYM medium (0.05, 0.1, 0.2, 0.5, 1 and 2 %, w/v).

To assess the effects of carbon (C) sources on enzyme production, the C source present in GYM medium (glucose, 1%, w/v) was replaced with various C sources (maltose, starch, lactose, fructose, tri-sodium citrate and rice bran; 1%, w/v each). To study the effects of nitrogen (N) sources, yeast extract (0.5%, w/v) was replaced with various organic and inorganic nitrogenous compounds (soybean meal, peptone, tryptone, beef extract, KNO_3 and NH_4Cl, 0.5% w/v each).

Screening of factors affecting protease production by PBD

PBD was used to screen signal factors among seven variables viz. rice bran, soybean meal, KH_2PO_4, $MgSO_4$, $CaCl_2$, inoculum size and inoculum age. The maximum and minimum ranges of the factors selected for protease production are given in Table 1. Design Expert 6 software (Stat-Ease) was used to generate a set of 12 experiments (Table 2). Protease production was set up in triplicates in 50 ml of the respective production medium and fermentation was carried out for 72 h (30°C, pH 10.7, 150 rpm). For each experiment, protease production was calculated in terms of protease units/ml (U/ml). The effect of each parameter on protease production was determined by calculating Student's t test and p-values. The effect of each factor was coded as positive or negative and significance was analyzed according to 't' and 'p' values [16].

S.No	Variable	High Level (+1)	Low Level (-1)
1	Rice bran (%, w/v)	2.0	0.2
2	Soybean meal (%,w/v)	3.0	0.2
3	KH_2PO_4 (% ,w/v)	2.0	0.5
4	$MgSO_4$ (%, w/v)	0.5	0.02
5	$CaCl_2$ (%, w/v)	0.1	0.0
6	Inoculum size (%, v/v)	5.0	0.1
7	Inoculum age (h)	24	8

Table 1: Maximum and minimum ranges of parameters selected for PBD.

Run	Rice bran	Soybean meal	KH_2PO_4	$MgSO_4$	$CaCl_2$	Inoculum size	Inoculum age
1	-1	-1	-1	+1	-1	+1	+1
2	-1	+1	+1	-1	-1	+1	-1
3	+1	-1	+1	+1	-1	-1	+1
4	-1	+1	-1	-1	+1	-1	+1
5	-1	+1	+1	+1	+1	-1	+1
6	+1	-1	-1	-1	+1	+1	+1

7	-1	-1	-1	-1	-1	-1	-1
8	+1	+1	-1	+1	-1	-1	-1
9	-1	-1	+1	+1	+1	+1	-1
10	+1	+1	-1	+1	+1	+1	-1
11	+1	-1	+1	-1	+1	-1	-1
12	+1	+1	+1	-1	-1	+1	+1

Table 2: Experimental design used in PBD.

Optimization of protease production by RSM

After PBD, RSM was used in the final round of medium optimization. Four parameters viz. rice bran, soybean meal, KH_2PO_4 and $CaCl_2$ were studied at five levels (-α, -1, 0, +1, +α) using the Central Composite Design (CCD). The experimental levels of the four parameters used in RSM in terms of actual and coded forms are listed in Table 3. A set of 30 experimental runs including six centre points were designed using Design Expert 6. Protease production was carried out in triplicates by dispensing 50 ml each of production media in 250 ml Erlenmeyer flasks. The media were inoculated with 5% (v/v, 0.5 OD_{600}) inocula and kept incubated in the shaker for 72 h (30° C, pH 10.7, 150 rpm). A multiple regression analysis of the data was carried out to obtain an empirical model that defines the response (protease production) in terms of the independent variables. The model equation is represented as:

$$Y=\beta_0+\sum \beta_i x_i +\sum \beta_i x_i^2 +\sum \beta_{ij} x_i x_j$$

Where, Y is the predicted response, β_0 is the intercept, β_i is the linear coefficient and β_{ij} is the interaction coefficient. The model was analyzed using ANOVA, 3D curves, contour and one-factor plots in order to study the interactions among various factors and to determine the optimum concentration of each for maximum protease production. Accuracy of the model was determined by validating the model.

Run	Variables	Range coding				
		+α	+1	0	-1	-α
1	Rice bran (%, w/v)	2	1.5	1.0	0.5	0
2	Soybean meal (%, w/v)	2.5	2.0	1.5	1.0	0.5
3	KH_2PO_4 (%,w/v)	0.9	0.7	0.5	0.3	0.1
4	$CaCl_2$ (%, w/v)	0.14	0.1	0.07	0.03	0

Table 3: Experimental ranges of four different variables used in CCD.

Statistical analysis of data

All the above experiments were repeated in triplicates and the final values have been presented as means ± S.D.

Results and Discussion

Isolation of bacteria

Nine (9) morphologically distinct bacterial strains were recovered. Of these six (6) strains showed proteolytic activity. Depending on the zone of clearance on skim milk agar medium, a strain (KW2) was selected for further studies. Strain KW2 forms round, smooth and light cream colonies with entire margins and convex elevations. The organism was gram positive, endospore forming, rod shaped and motile. It showed positive results for catalase, oxidase, gelatin liquefaction, and starch hydrolysis tests but negative for Tween 20/80 hydrolysis. KW2 could grow at pH 8-12 with optimal growth at pH 10. It could tolerate up to 5% NaCl (w/v) and grew well at the temperature range of 25-45°C with optimum at 30°C. The phenotypic features of KW2 corresponded with those outlined in description of the genus *Bacillus* in the Bergey's Manual of Determinative Bacteriology [17].

Optimization by OVAAT approach

Protease production by *Bacillus* sp. KW2 increased progressively with increase in incubation temperature and peaked at 30°C (246 ± 16 U/ml). Protease production declined beyond 30°C; at 40°C, enzyme production was reduced to 50 ± 6 U/ml (Figure 1).

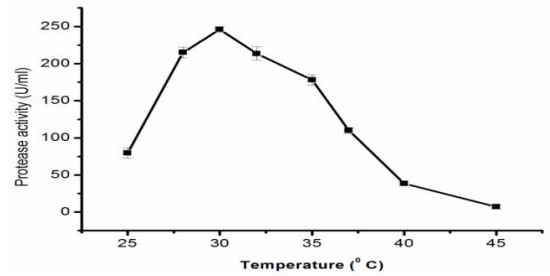

Figure 1: Effects of incubation temperature on protease production by strain KW2.

Optimum production of protease at 30°C was also reported for *Bacillus* sp. B21-2 [18], *Bacillus* sp. MIG [19] and *Bacillus pantothenticus* [20]. Sodium carbonate was found to significantly affect medium pH and protease production by *Bacillus* sp. KW2. Maximum protease production was observed at 1% (w/v) Na_2CO_3 concentration. Presence of Na_2CO_3 (1% w/v) maintains pH of the medium at around 10.7 (Figure 2). Similarly, Horikoshi also used Na_2CO_3 (1% w/v) to adjust pH of the medium to alkaline levels for growth and alkaline protease production by *Bacillus* No 221 [21]. However, *Bacillus horikoshii* [22] and *Bacillus circulans* MTCC 7942 [23] were reported to produce alkaline protease optimally in presence of 0.6% (w/v) Na_2CO_3. During protease production by alkaliphiles, Na_2CO_3 incorporation as a source of alkalinity to their growth media is routinely practiced so as to simulate their growth [23]. The role of Na_2CO_3 is not only to maintain alkalinity of the medium but also to provide Na^+ ions required for growth of the alkaliphiles.

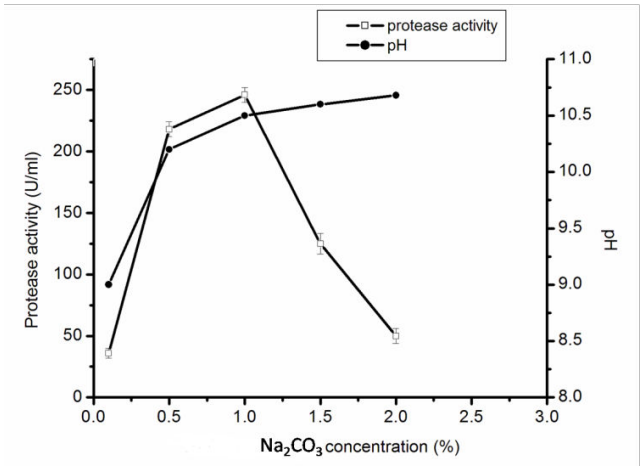

Figure 2: Effects of Na_2CO_3 concentration on pH of the medium and protease production.

Effects of the carbon sources on protease production by *Bacillus* sp. KW2 were studied by using various C sources instead of glucose in GYM medium. Enhancement of protease production was observed in presence of rice bran (496 ± 4 U/ml), fructose (365 ± 5 U/ml), and starch (364 ± 8 U/ml). However, lactose and tri-sodium citrate inhibited protease production with resultant titer values of 112 ± 5 U/ml and 100 ± 4 U/ml respectively (Figure 3).

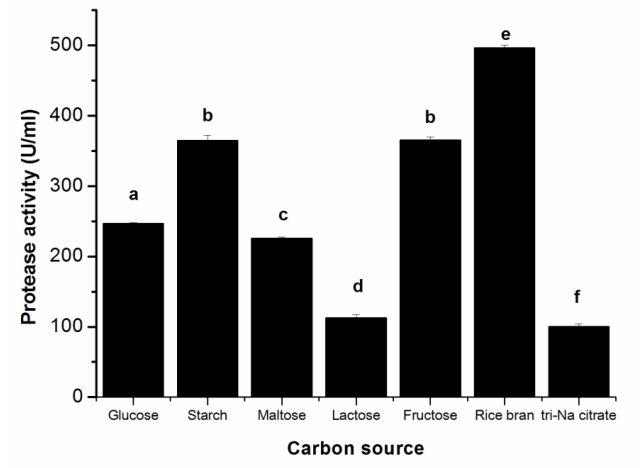

Figure 3: Effects of carbon sources on protease production. Statistical significance was determined by student's t test at $p \leq 0.05$. Values with same alphabets are not statistically significant.

Enhancement of protease production in presence of rice bran is an interesting feature because rice bran is a by-product of the rice milling industry that can be used as cheap and readily available substrate for enzyme production. Organic nitrogen sources such as soybean meal, yeast extract and peptone are well known inducers of protease production. In this study, soybean meal was found to be the best nitrogen source for enhanced protease production by the strain KW2 (Figure 4). Similar results were also reported by Joo et al. [24] indicating enhanced protease production by soybean meal in *Bacillus clausii*. However, inorganic nitrogen sources such as KNO_3 and NH_4Cl inhibited protease production by KW2. The inhibitory effects of inorganic nitrogen sources on protease production have also been reported for *Bacillus* sp. JB-99 [25], *Bacillus horikoshii* [4], *Bacillus clausii* [26] and *Streptomyces* sp. [27]. After optimization by OVAAT approach, the GYM medium was modified. The modified medium was composed of rice bran (1% w/v), soybean meal (0.5%, w/v), $MgSO_4$ (0.5%, w/v), KH_2PO_4 (0.5 %, w/v) and Na_2CO_3 (1.0%, w/v).

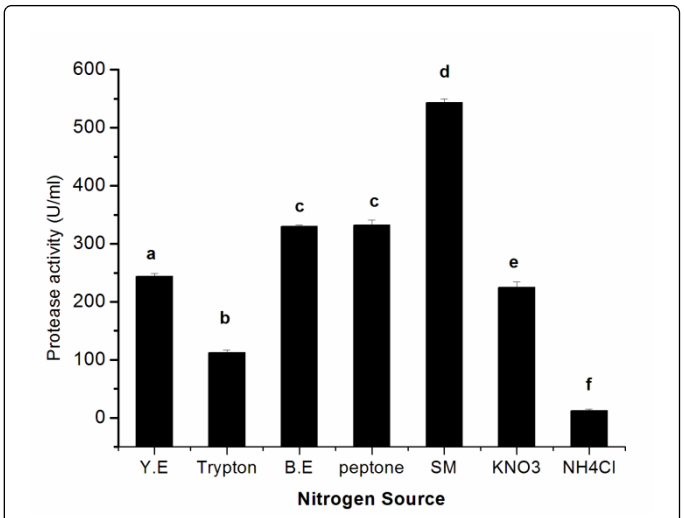

Figure 4: Effects of nitrogen sources on protease production: Yeast extract (Y.E), Beef extract (B.E), Soybean meal (S.M). Statistical significance was determined by student's t test at $p \leq 0.05$. Values with same alphabets are not statistically significant.

Optimization by statistical approaches

PBD: A total of seven variables were studied by using PBD. Higher levels of rice bran, soybean meal, inoculum age and inoculum size enhanced protease production whereas higher level of KH_2PO_4 reduced protease production (Table 4). Effect of divalent metal ions (Ca^{+2} and Mg^{+2}) on protease production were also investigated. In this study, Ca^{+2} was found to be stimulatory for protease production; however, high level of Mg^{+2} (0.5%, w/v) had inhibitory effect on protease production. Ca^{+2} and Mg^{+2} were reported to be essential for protease production by *Bacillus* sp. N-40 [28]. In contrast to the present study, Tiwary and Gupta [16] reported that keratinase production by *Bacillus licheniformis* was inhibited by Ca^{+2} while Mg^{+2} did not exert any significant effect. Four parameters (rice bran, soybean meal, KH_2PO_4 and $CaCl_2$) were selected for further optimization studies. $MgSO_4$ was kept at lower level (0.02 w/v) while inoculum age and inoculum size were fixed at higher levels (24h and 5%, v/v respectively). Four parameters (rice bran, soybean meal, KH_2PO_4 and $CaCl_2$) were selected for further optimization studies. $MgSO_4$ was kept at lower level (0.02 w/v) while inoculum age and inoculum size were fixed at higher levels (24h and 5%, v/v respectively). Four parameters (rice bran, soybean meal, KH_2PO_4 and $CaCl_2$) were selected for further optimization studies. $MgSO_4$ was kept at lower level (0.02 w/v) while inoculum age and inoculum size were fixed at higher levels (24h and 5%, v/v respectively).

Factors	Mean total protease production (U/ml) at +1 level	Mean total protease production (U/ml) at -1 level	Student t-Test	P-value	Remarks
Rice bran	1476	714	+218	<0.0001	Positive
Soybean meal	1231	960	+33	<0.0001	Positive
KH_2PO_4	514	1650	-75	<0.0001	Negative
$MgSO_4$	898	1293	-24	<0.0001	Negative
Inoculum age	1197	994	+24	<0.0001	Positive
Inculum size	1139	1081	+1.9	<0.001	Positive
$CaCl_2$	1254	937	+20	<0.001	Positive

Table 4: Screening of signal parameters for protease production by PBD.

Optimization by RSM

The effects of four parameters (rice bran, soybean meal, KH_2PO_4 and $CaCl_2$ concentrations) on protease production were studied using RSM. A set of 30 experiments with different combinations of the four selected factors were performed. Both observed and predicted values were found to be similar suggesting the authenticity of the model. The results of the RSM experiments for KW2 are presented in Table 5.

The protease production (Y) by *Bacillus* sp. KW2 can be expressed in terms of the following regression equation:

$$Y= +1178.00+47.29.A -131.21. B- 16.29. C+18.13. D-260.28. A^2-177.91.B^2-208.66.C^2 - 156.28.D^2 -51.3 .A.B +23.31.A.C+10.3. A .D -26.31.B.C$$

Where A, B, C and D represent rice bran, soybean meal, KH_2PO_4 and $CaCl_2$ concentrations respectively. The model coefficients determined by multiple linear regression and analysis of variance (ANOVA) are presented in Table 6. The Model F-value of 33.7 implies that the model is significant. There is only a 0.01% chance that "Model F-Value" this large could occur due to noise. Values of "Prob>F" less than 0.05 indicate that model terms are significant. R^2 value (0.959) is high indicating that the model is significant. The predicted R^2 of 0.8515 is in reasonable agreement with the adjusted R^2 of 0.9312. "Adeq Precision" measures the signal to noise ratio. A ratio greater than 4 is desirable. The ratio of 17.713 obtained in ANOVA table indicates an adequate signal. One factor, contour and 3D response surface curves were plotted to study interactions among the four factors to determine the optimum concentration of each factor for maximum protease production. Based on the predictions drawn from the one factor plot, rice bran and KH_2PO_4 at their maximum and minimum levels were inhibitory for enzyme production. Higher level of soybean meal inhibited protease production whereas $CaCl_2$ did not have significant effect on protease production. The interactions among the variables are shown in Figures 5A-5D. Higher level of soybean meal inhibited protease production whereas $CaCl_2$ did not have significant effect on protease production. The interactions among the variables are shown in figures 5A-5D.

	Rice Bran	Soybean meal	KH_2PO_4	$CaCl_2$	Actual protease production (U/ml)	Predicted protease Production (U/ml)
1	0	0	0	+α	686	589.3
2	-1	+1	+1	-1	112	173.96
3	0	0	0	0	1250	1178
4	-1	+1	-1	+1	256	321.42
5	+1	-1	-1	-1	560	542.92
6	+1	+1	-1	-1	160	230.5
7	+1	+1	+1	+1	203	248.79
8	-1	-1	-1	-1	380	412.96
9	0	0	0	0	1026	1178.0
10	-1	-1	+1	-1	476	386.37
11	0	0	0	+1	1156	1178.0
12	+1	+1	-1	+1	201	287.37
13	0	0	-α	0	468	375.96
14	0	+α	0	0	376	203.96
15	0	0	0	0	1312	1178.0
16	+1	-1	+1	-1	606	609.58
17	0	0	0	+1	1112	1178.0
18	0	- α	0	0	680	728.79
19	0	0	0	0	1212	11787.0
20	+1	-1	-1	+1	545	599.79
21	+α	0	0	0	334	231.46
22	-α	0	0	0	63	42.9
23	-1	-1	-1	+1	456	428.58
24	+1	-1	+1	+1	654	666.46
25	-1	+1	+1	+1	166	189.58
26	0	0	+α	0	342	310.79
27	-1	+1	-1	-1	264	305.79
28	+1	+1	+1	-1	120	191.92
29	0	0	-1	-α	543	516.63
30	-1	-1	+1	+1	346	402.0

Table 5: Results of RSM studies for protease production by strain KW2.

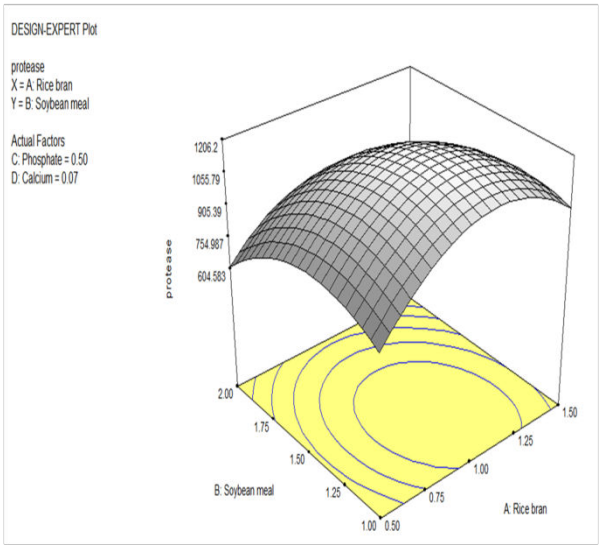

Figure 5: 3D response surface curve showing interaction effects of variables on protease production (A-D) by *Bacillus* sp. KW2. Out of four variables, interactions between two variables were studied by keeping two variables at their optimum levels as selected from the RSM study. **Figure 5A:** Interactions between rice bran and soybean meal at fixed $CaCl_2$ (0.07%) and KH_2PO_4 (0.5%) concentrations.

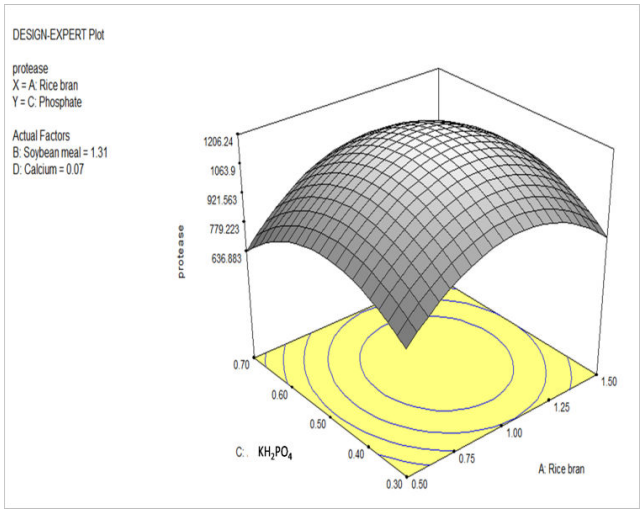

Figure 5B: Interactions between rice bran and KH_2PO_4 at fixed soybean meal (1.31%) and $CaCl_2$ 0.07%) concentrations.

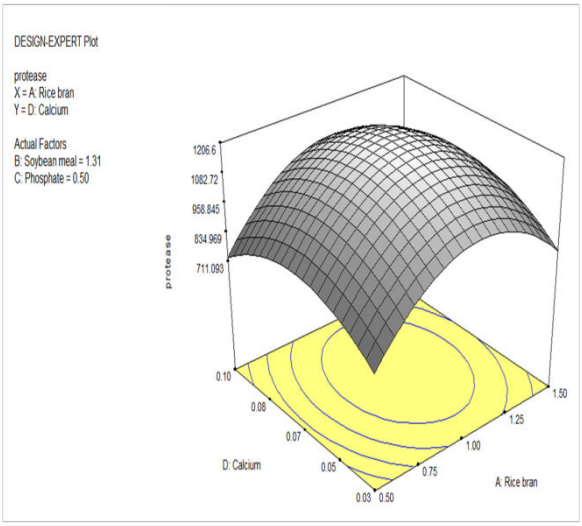

Figure 5C: Interactions between $CaCl_2$ and rice bran at fixed soybean meal (1.31%) and KH_2PO_4 (0.5%) concentrations.

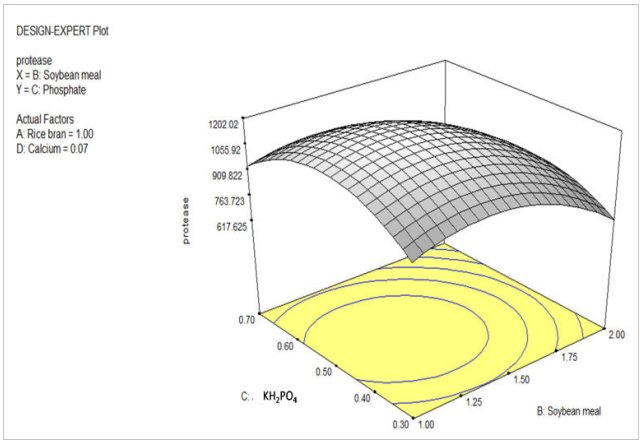

Figure 5D: Interactions between KH_2PO_4 and soybean meal at fixed rice bran (1.0%) and $CaCl_2$ (0.07%) concentrations.

On the basis of the overall predictions and the interaction plots, it was observed that maximum protease production is achieved when concentrations of rice bran, KH_2PO_4 and $CaCl_2$ were kept at their central levels (i.e. 1%, 0.5% and 0.07%, w/v respectively) and soybean meal at 1.3% (w/v). Rice bran contains 49.4% carbohydrates, 21.3% fat and 16.5% protein [29]. In India and other countries, approximately one million tons of rice bran are produced annually and used predominantly for animal feed [30]. Use of rice bran as substrate for microbial enzyme production has been reported earlier especially for solid state fermentation [31-33]. There are meagre reports on use of rice bran in submerged fermentation. And there are no reports so far on use of rice bran in RSM studies. This study will be the first report on use of rice bran in optimization of alkaline protease production by statistical approaches. The accuracy of the values predicted by the model was verified by 10 additional independent experiments. The

predicted and experimental values of protease production are presented in Table 7.

F-Value	33
P>F	0.0001
Mean	535
R^2	0.9597
Adjusted R^2	0.9312
Predicted R^2	0.8515
Coefficient of variance	18.19
Adequate precision	17.7

Table 6: ANOVA values of the CCD model.

The experimentally determined values were in close agreement with the predicted values showing the authenticity of the model. Based on the response surface curves and validation experiments, the final optimized medium for protease production by *Bacillus* sp. KW2 was as follows: rice bran (1%, w/v), soybean meal (1.3%, w/v), KH_2PO_4 (0.5%, w/v), $CaCl_2$ (0.07%, w/v), $MgSO_4$ (0.02%,w/v) and Na_2CO_3 (1.0%, w/v). The Final Optimized Medium was designated as FOM. FOM is a relatively cheaper medium as compared to GYM. Protease production by strain KW2 was recorded as 246 ± 5 U/ml in the unoptimized medium (GYM) and 1302 ± 56 U/ml in the FOM medium. Overall, a 4.8 fold increase in protease production was achieved after optimization. Similar fold increase in protease production after optimization by statistical methods were reported earlier for *Bacillus mojavensis* (4.2 fold) [34], *Microbacterium* sp. (3.6 fold) [12] and *Bacillus amyloliquefaciens* (3.92 fold) [35]. However, higher fold increase in protease production have also been reported for *Bacillus* sp. BGS (6.36 fold) [8] and *Bacillus* sp. RG-14 (12.8 fold) [36].

Run	Rice bran (%, w/v)	Soybean meal (%, w/v)	KH_2PO_4 (%, w/v)	$CaCl_2$ (w/v, %)	Predicted Protease production (U/ml)	Observed Protease production (U/ml)
1	1	1.3	0.5	0.07	1201	1302
2	0.5	1.5	0.5	0.07	868	946
3	0.7	1.5	0.5	0.07	1054	1102
4	1.5	1.5	0.5	0.07	965	896
5	1	1.0	0.5	0.07	1175	1265
6	1	1.5	0.5	0.07	1177	1186
7	1	2	0.5	0.07	868	745
8	1	1.7	0.5	0.07	1096	948
9	0.2	1.5	0.5	0.07	433	467
10	1	0.5	0.5	0.07	728	686

Table 7: Validation of the CCD model.

Studies of time course for protease production by *Bacillus* sp. KW2 indicated that protease production started at the exponential phase and reached maximal levels at the late stationary phase of growth (84 h) (Figure 6). Similar to our findings, production of protease at late stationary phase have also reported for *Bacillus cereus* at 96 h incubation [37] and a marine actinomycete, *Saccharopolyspora* sp. at 14 days incubation [38].

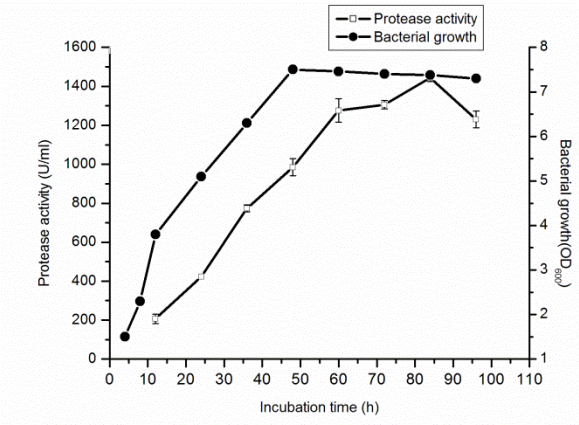

Figure 6: Time course of growth and protease production of *Bacillus* sp. KW2 in FOM medium. Bacterial growth was monitored by measuring the OD at 600 nm.

Conclusions

The present study shows that protease production is greatly influenced by cultural conditions and media constituents. The optimum conditions for protease production by *Bacillus* sp. KW2 was observed at 30°C, pH 10.7 and 84 h of incubation. This study also established that the strain KW2 could produce alkaline protease in a low cost medium (FOM) containing rice bran and soybean meal as major carbon and nitrogen sources. Protease production was also optimized at two levels i.e. one-variable-at-a-time and statistical approaches using Design Expert 6 software. After optimization, protease production was increased by 4.8 fold. Strain KW2 may be a promising candidate for production of alkaline protease for commercial applications.

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Disclosure statement

Authors do not have any competing or conflicting interests in submission of this manuscript.

References

1. Kumar CG, Takagi H (1999) Microbial alkaline proteases: from a bioindustrial viewpoint. Biotechnol Adv 17: 561-594.
2. Nilegaonkar SS, Kanekar PP, Sarnaik SS, Kelkar AS (2002) Production, isolation and characterization of extracellular protease of an alkaliphilic strain of *Arthrobacterramosus*, MCM B-351 isolates from the alkaline lake of Lonar, India. World J Microbiol Biotechnol 18: 785-789.
3. Rao MB, Tanksale AM, Ghatge MS, Deshpande VV (1998) Molecular and biotechnological aspects of microbial proteases. Microbiol Mol Biol Rev 62: 597-635.
4. Joo HS, Kumar CG, Park GH, Kim KT, Paik SR, (2002) Optimization of the production of an extracellular alkaline protease from *Bacillus Horikoshi*. Process Biochem 38: 155-159.
5. Haddar A, Fakhfakh-Zouari N, Hmidet N, Frikha F, Nasri M (2010) Low-cost fermentation medium for alkaline protease production by *Bacillus mojavensis* A21 using hulled grain of wheat and *sardinella* peptone. J Biosci Bioeng 3: 288-294.
6. Oskouie SFG, Tabandeh F, Yakhchali B, Eftekhar F (2008) Response surface optimization of medium composition for alkaline protease production by *Bacillus clausii*. Biochem Eng J 39: 37-42.
7. Dutta JR, Dutta PK, Banerjee R (2004) Optimization of culture parameters for extracellular protease production from a newly isolated *Pseudomonas* sp. using response surface and artificial neural network models. Process Biochem 39: 2193-2198.
8. Moorthy IMG, Baskar R (2013) Statistical modeling and optimization of alkaline protease production from a newly isolated alkaliphilic *Bacillus* species BGS using response surface methodology and genetic algorithm. Prep Biochem Biotech 43: 293-314.
9. Naidu KSB, Devi KL (2005) Optimization of thermostable alkaline protease production from species of *Bacillus* using rice bran. Afr J Biotechnol 4: 724-726.
10. De Azeredo LA, De Lima MB, Coelho RR, Freire DM (2006) A low-cost fermentation medium for thermophilic protease production by *Streptomyces* sp. 594 using feather meal and corn steep liquor. Curr Microbiol 53: 335-339.
11. Joo HS, Chang CS (2005) Production of protease from a new alkaliphilic *Bacillus* sp I-312 grown on soybean meal: Optimization and some properties. Process Biochem 40: 1263-1270.
12. Thys RCS, Guzzon SO, Cladra-Olivera F, Brandelli A (2006) Optimization of protease production by *Microbacterium* sp. in feather meal using response surface methodology. Process Biochem 41: 67-73.
13. Mukherjee AK, Adhikai H, Rai SK (2008) Production of alkaline protease by a thermophilic *Bacillus subtilis* under solid-state fermentation (SSF) condition using *Imperata cylindrica* grass and potato peel as low-cost medium: Characterization and application of enzyme in detergent formulation. Biochem Eng J 39: 353-361.
14. Prakasham RS, Rao ChS, Sarma PN (2006) Green gram husk--an inexpensive substrate for alkaline protease production by *Bacillus* sp. in solid-state fermentation. Bioresour Technol 97: 1449-1454.
15. Horikoshi K (2004) Alkaliphiles. Proc Jpn Acad SerB 80: 166-178.
16. Tiwary E, Gupta R (2010) Medium optimization for a novel 58 kDa dimeric keratinase from *Bacillus licheniformis* ER-15: biochemical characterization and application in feather degradation and dehairing of hides. Bioresour Technol 101: 6103-6110.
17. Holt JK, Krieg NR, Sneath PHA, Staley JT (1994) Bergey's Manual of Determinative Bacteriology. (9th edn.), Williams & Wilkins, Baltimore, MS, USA.
18. Fujiwara N, Yamamoto K (1987) Production of alkaline protease in a low-cost medium by alkalophilic *Bacillus* sp. and properties of the enzyme. J Ferment Technol 65: 345-348.
19. Gouda MK (2006) Optimization and purification of alkaline proteases produced by marine *Bacillus* sp. MIG newly isolated from eastern harbour of Alexandria. Pol J Microbiol 55: 119-126.
20. Shikha Sharma A, Darmwal SN (2007) Improved production of alkaline protease from a mutant of alkaliphilic *Bacillus pantotheneticus* using molasses as a substrate. Bioresour Technol 98: 881-885.
21. Horikoshi K (1971) Production of alkaline enzymes by alkaliphilic microorganisms Part I. Alkaline protease produced by *Bacillus* No. 221. Agr Biol Chem 35: 1407-1414.
22. Joo HS, Choi JW (2012) Purification and characterization of a novel alkaline protease from *Bacillus horikoshii*. J Microbiol Biotechnol 22: 58-68.
23. Patil U, Chaudhari A (2013) Production of alkaline protease by solvent-tolerant alkaliphilic *Bacillus circulans* MTCC 7942 isolated from hydrocarbon contaminated habitat: process parameters optimization. ISRN Biochemistry 2013: 10.
24. Joo HS, Kumar CG, Park GC, Paik SR, Chang CS (2003) Oxidant and SDS-stable alkaline protease from *Bacillus clausii* I-52: production and some properties. J Appl Microbiol 95: 267-272.
25. Johnvesly B, Naik GR (2001) Studies on production of thermostable alkaline protease from thermophilic and alkaliphilic *Bacillus* sp. JB-99 in a chemically defined medium. Process Biochem 37: 139-144.
26. Kumar CG, Joo HS, Koo YM, Paik SR, Chang CS (2004) Thermostable alkaline protease from a novel marine haloalkalophilic *Bacillus clausii* isolate. World J Microbiol Biotechnol 20: 351-357.
27. Lazim H, Manka H, Slama N, Barkallah I, Liman F (2009) Production and optimization of thermophilic alkaline protease in solid state fermentation by *Streptomyces* sp CN 902. J Ind Microbiol Biotechnol 36: 531-537.
28. Sevnic N, Demirkan E (2011) Production of protease by *Bacillus* sp. N-40 isolated from soil and its enzymatic properties. J Biol Environ Sci 5: 95-103.
29. Rao BSN (2000) Nutritive values of rice bran. National Institute of Nutrition, India.
30. Faria SADSC, Bassinello PZ, Penteado MDVC (2012) Nutritional composition of rice bran submitted to different stabilization procedures. Braz J Pharm Sci 48: 651-657.
31. Ali SS, Vidhale NN (2013) Protease Production by *Fusarium oxysporum* in Solid- State Fermentation Using Rice Bran. Am J Microbiol Res 1: 45-47.
32. Sumantha A, Deepa P, Sandhy C, Szakacs G, Soccol CR (2006) Rice bran as a substrate for proteolytic enzyme production. Braz Arch Biol Technol 49: 843-851.
33. Chutmanop J, Chuichulcherm S, Chisti Y, Srinophakun P (2008) Protease production by *Aspergillus oryzae* in solid-state fermentation using agroindustrial substrates. J Chem Technol Biotechnol 83: 1012-1018.
34. Beg QK, Sahai V, Gupta R (2003) Statistical media optimization and alkaline protease production from *Bacillus mojavensisin* a bioreactor. Process Biochem 39: 203-209.
35. Cheng SW, Wang YF, Wang ML (2012) Statistical optimization of medium compositions for alkaline protease production by newly isolated *Bacillus amyloliquefaciens*. Chem Biochem Eng Q 26: 225-231.
36. Chauhan B, Gupta R (2004) Application of statistical experimental design for optimization of alkaline protease production from *Bacillus* sp. RGR-14. Process Biochem 39: 2115-2122.
37. Uyar F, Porsuk I, Kizil G, Yilmaz EI (2011) Optimal conditions for production of eztracellular protease from newly isolated *Bacillus cereus* strain CA 15. EurAsian J Biosc 5: 1-9.
38. Raut GR, Chakraborty S, Chopade BA, Kokare CR (2013) Isolation and characterization of organic solvent stable protease from alkaliphilic marine *Saccharopolyspora*. IJMS 42: 131-138.