JOURNAL OF PURE AND APPLIED MICROBIOLOGY, August 2014. Vol. **8**(4), p. 2523-2533

Process Development to Augment the Production of Microbial Extra Cellular Protease using Response Surface Methodology

Muhammad Asif[1], Taskeen Niaz[2], Arif Malik[2], Ahmad Zaheer[3], Abrar Hussain[1], Humera Aslam Awan[4] and Mahmood Rasool[5]*

[1]Department of Biotechnology and Informatics, BUITEMS, Quetta, Pakistan.
[2]Institute of Molecular Biology & Biotechnology (IMBB), the University of Lahore, Pakistan.
[3]National Institute for Biotechnology & Genetic Engineering (NIBGE), Faisalabad, Pakistan.
[4]Department of Biological Sciences, Forman Christian College University, Lahore, Pakistan.
[5]Center of Excellence in Genomic Medicine Research (CEGMR), King Abdulaziz University, Jeddah, Saudi Arabia.

(Received: 03 February 2014; accepted: 21 March 2014)

Alkaline proteases are the most important group of extremoenzymes which have a wide array of applications in various industries such as detergent, food, pharmaceutical, leather and silk. For the purpose of maximum alkaline protease production, response surface methodology was performed using *Bacillus licheniformis*. The sources used were glucose as carbon source, peptone as nitrogen source, sodium phosphate as phosphorus source and zinc sulfate and magnesium sulfate as micronutrients salt solution; pH and temperature selected for optimization. Response surface methodology central composite deigns, involving 2^6 full factorial half fractions, designed 53 experiments with different combination using six sources and analysis of data was done by SPSS 16. The multiple linear regression model equation was articulated by the coefficient of the determination R^2 and statistical significance of the variables were checked by p (<0.05) value, if lesser the p value then higher the significant of the subsequent coefficient. Importance of the regression coefficient was tested by *t* test. Applying statistical analysis multiple linear regression using quadric equation shows that the effect of carbon (C), micronutrient salt solutions (M), phosphorous (P^2), carbon and phosphorous (CP), carbon and micronutrient salt solutions (CM), carbon and temperature (CT), nitrogen and micronutrient salt solutions (NM), nitrogen and temperature (NT), micronutrient salt solutions and pH (MpH), were given significant results and enhance the production of alkaline proteases by *Bacillus licheniformis*.

Keywords: Alkaline Protease, Response Surface Methodology, *Bacillus licheniformis*.

Proteases are the most important group of industrial enzymes that are used in different processes, i.e. in the detergent and food industries[1], pharmaceutical, leather processing[2-3], cosmetic, brewing, silk, recovery of silver from used x-ray films and for treatment of house hold wastes[4-6]. The molecular weight of proteases ranges from 18 – 90 kDA[7]. Proteases dominate the worldwide enzyme market, accounting for a two-third share of the detergent industry[8].

* To whom all correspondence should be addressed.
Tel: +966-6401000 ext 25479; Fax: +966-6952521;
E-mail: mahmoodrasool@yahoo.com

Microorganisms are the major source for enzyme production[9-10] and the genus Bacillus are one of the major and most omnipresent genera of bacteria, which contains 65 species, and the new species continually being described. The genus covers all aerobic or facultative anaerobic, spore-forming, rod-shaped bacteria, and is regularly encountered and cultivated from soil samples as their primary habitat[11]. The importance of the *Bacillus* strains is due to production of important enzymes in a very short time of period into the fermentation broth[2].

Recently, no defined medium has been established for the optimum production of protease from different microbial sources because each organism has different cultivation conditions for maximum protease production[2].

It is well known that extra cellular protease production in microorganisms is greatly influenced by media components, especially carbon and nitrogen sources, physical factors, such as pH, temperature, inoculum density, dissolved oxygen and incubation time[12-14]. Proteases possess some characteristics of biotechnological interest due to these have become the most important industrial enzymes. Almost all proteases are heat resistance, varies widely in their specific activities, optimum pH, pH stability range, heat sensitivity, active site, and catalytic mechanism and stability profiles[3].

Response surface methodology (RSM) is a combination of mathematical and statistical approach to study the characteristics of the given system, design experiments, building models, evaluating the effects of factors and searching for the optimum conditions. It has successfully been used in the rational optimization of several bioprocesses[2,11,15]. The conventional method of optimization involves varying one parameter at a time and keeping the others constant. This often does not bring about the effect of interaction of various parameters as compared to factorial design. RSM is a useful model for studying the effect of several factors influencing the responses by varying them simultaneously and carrying out a limited number of experiments[2,14]. RSM has been extensively applied in optimization of medium composition, conditions of enzymatic hydrolysis, fermentation and food manufacturing process. It is the collection of statistical techniques for experiment design, model development, evaluation factors and optimum conditions search[5,16].

The aim of this study was to improve the production of alkaline proteases by using *Bacillus licheniformis*. The optimization of medium component sources were carbon, nitrogen, Phosphorus, Micronutrient salt solutions, pH and temperature play a very significant role in increasing the production of alkaline protease. The use of experimental design response surface methodology successfully applied in this study. We designed and performed 53 experiments through central composite design model, two factorial (half fraction) with 06 factors, cube points 32, center points in cube 09 along with 12 axial points

MATERIALS AND METHODS

Organism

Alkaline proteases producing bacterial strain was obtained from Pakistan type culture collection (PTCC) of Food Biotechnology and Research Centre PCSIR Laboratories, Lahore. It was being maintained on nutrient agar slants and stored at 4 ºC in a refrigerator.

Detection, isolation and screening of protease enzyme activity

For the purpose to detect the alkaline protease producing strain detection, media was prepared (10 gm skimmed milk/100ml, 10gm agar/ 100ml and phosphate buffer 0.2 M (300ml) maintained pH 7.0).

The above mentioned medium components were autoclaved at 110 °C for 15 min separately to avoid the coagulation and then after removing from autoclave cooled the media components and then mixed all media components with each other under sterile conditions. The bacterial colonies which appeared on the nutrient agar plates containing protease producing strains pour on skimmed milk medium plates. Then the plates were incubated at 37°C for 48 hour. After the incubation period the plates were flooded with 10 % tannic acid. Protease producing colonies showed the hollow zones around them showing digestion of proteins by the proteases produced by the bacteria, shows the presence of protease enzyme detection.

Inoculum preparation

A 24-hour-old loopful culture was being transferred into the sterilized inoculum medium consisting of (in g/L): glucose 5.0, soybean meal 10.0, K_2HPO_4 3.0, $MgSO_4 \cdot 7\ H_2O$ 0.5, NaCl 0.5 and $CaCl_2 \cdot 2H_2O$ 0.5. The inoculated broth was incubated in water bath shaker for 24 h at 37°C for the propagation of bacteria up to 108–1010 cells/ mL. Then, 1 % (by volume) of this inoculum medium was used to inoculate the growth medium for alkaline protease production[17].

Shake flask experiment or cultivation of growth medium

A volume of 100ml for the growth medium

containing glucose as C source , peptone used as N source , Na_2PO_4 used as phosphorus source,) and $ZnSO_4$ and $MgSO_4$ as micronutrient salt solution, pH and temperature (ml/L) with different concentrations according to designed experiments in 250ml flask, set pH according to experimental design for *Bacillus lichenifromis* (the pH values was adjusted by adding 1N NaOH and 1N HCl) then autoclave at 121°C for 15min and then remove from autoclave, cool for 10 minutes at room temperature and was inoculated with 1ml of the inoculated medium and then incubated in shaking incubator for 24 hours at 45°C for at 120 rpm.

Media optimization

To optimize media components the following different factors were studied i.e. carbon source, nitrogen source, phosphorus source, micronutrients, pH and temperature (Table 1).

Protease Assay for measure enzymatic activity

To measure the enzymatic activity, Kunitz (1947) method is used[18]. Firstly, phosphate buffer solution (K_2HPO_4 1.74gm/100ml and KH_2Po_4 1.36gm/100ml) was prepared in distilled water and then 1% casein added and maintained pH 7. Secondly prepared solution of 10% TCA in 100ml distilled water then added 2ml casein solution, at the end 1ml enzyme solution was added in all test tubes. Then all these test tubes were incubated at 30°C for 20min. After incubation, added 3ml of 10% TCA solution in all test tubes. All test tubes were centrifuged for 10 min at 5000 rpm. After centrifugation, removed the supernatants and measured the enzymatic activity at spectrophotometer at 280 nm. One unit of protease was equivalent to the amount of enzyme required to release 1µg of tyrosine/ml/min under assay conditions.

Experimental design and optimization by RSM

Central composite experimental design adopted for the optimization and improving total protease production of Bacillus *lichenifromis* species. Two level factorial with half fraction included 6 factors 2^6, cube points 32 with center points in cube 9, axial points 12, center points in axial 0 with alpha:2.37 leading to a total number of 53 experiments was performed (Table 2). Carbon concentration w/v (X_1 g/L), nitrogen concentration w/v (X_2 g/L), phosphorus concentration w/v (X_3 g/L), and micronutrients salt solutions v/v (X_4 ml/L), pH (X_5) and temperature (X_6), selected as independent variables and depended variables *Bacillus licheniformis* Y_1. The maximum and minimum range of independent variables investigated and full experimental plan with respect to their actual values and coded forms listed in table 2 and 3.

A second degree of quadric polynomial equation is selected to estimate the response of dependent variables. The polynomial equation is then fitted to the data by the multiple linear regression. The empirical model that related the response measured to the independent variables of the experiment. A six factor system, the model equation is

$$Y= \beta_0+ \beta_1X_1+ \beta_2X_2+ \beta_3X_3 + \beta_3X_3++ \beta_4X_4+ \beta_5X_5 \beta_5X_6+\beta_{11}X^2_1 +\beta_{22} X^2_2+ \beta_{33} X^2_3+ \beta_{44} X^2_4+ \beta_{55} X^2_5+ \beta_{66} X^2_6+\beta_{12}X_1X_2+ \beta_{13}X_1X_3+ \beta_{14}X_1X_4+ \beta_{15}X_1X_5+ \beta_{16}X_1X_6+\beta_{23}X_2X_3+ \beta_{24}X_2X_4+ \beta_{25}X_2X_5+ \beta_{26}X_2X_6 \beta_{34}X_3X_4+ \beta_{35}X_3X_5+ \beta_{36}X_3X_6+ \beta_{45}X_4X_5+ \beta_{46}X_4X_6+ \beta_{56}X_5X_6$$

Y predicted response, $X_1+X_2+X_3+X_4+X_5+X_6$ are independent variables, β_0 is intercept, β_1, β_2, β_3, β_4, β_5 are linear coefficients, β_{11}, $\beta_{22,}$ β_{33}, β_{44}, β_{55}, β_{66} squared coefficients and β_{12} β_{13}, β_{14},β_{15}, β_{16},β_{23}, β_{24}, β_{25}, β_{26}, β_{34}, β_{35}, β_{36}, β_{45}, β_{46}, β_{56}, are interaction coefficients.

Using the above model to obtain the optimum concentration of the medium components, we designed our experimental model in SPSS statistical software and also analyzed date through this software. We checked the effect of maximum and minimum concentrations C, N, P, micronutrients salt solution, pH and temperature of independent variables on dependent variables on Bacillus *licheniformis* through draw response surface graphs. All response surface graphs draw in STATISCA 5.5 version software.

RESULTS

Results of central composite design with a total number of 53 experiments studying the effects of six independent variables, viz., C, N, P, micronutrients salt solution, pH and temperature concentrations on protease production are presented in table 4. Statistical analysis was done by the Fisher's statistical test for analysis of variance (ANOVA); results are shown in table 5.

The regression equation obtained after analysis of variance (ANOVA) gives the F=5.105

Table 1. The minimum and maximum values of selected factors and their sources

S.No	Name of Factors	Sources	Max and min range/Concentrations
1	Carbon	Glucose	1-5 g/100ml
2	Nitrogen	Peptone	0-4 g/100ml
3	Phosphorus	Na_2PO_4	0.5-4.25 g/100ml
4	Micronutrients	$ZnSo_4$ and $MgSo_4$	0.1-1 ul/100ml
5	pH		5-8
6	Temperature		16-42

demonstrate that the model is highly significant for regression model. The goodness of the model was checked by coefficient of determination, R^2, which was found be to 0.857, indicates that 85.7% of the variability in the response could be expressed by the model. The value of adjusted determination coefficient (Adj. R^2=0.689) indicates the significant of the model. Value of the correlation coefficient (R=0.926) indicates the higher significant correlation between independent variables. The p-values are used as a tool to check the significance of each coefficient, which also indicate the interaction strength between each independent variable. Smaller the p-values, bigger

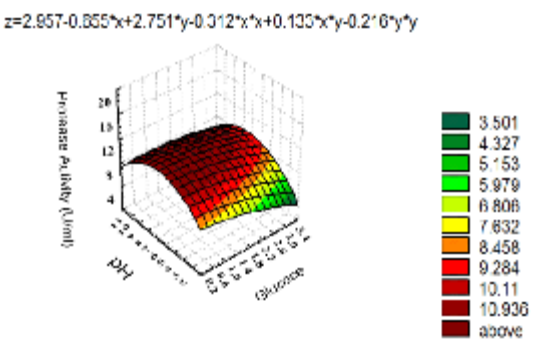

Fig. 1. Response surface plot showing the effect of pH and glucose concentration on the production of alkaline protease activity by *Bacillus licheniformis*

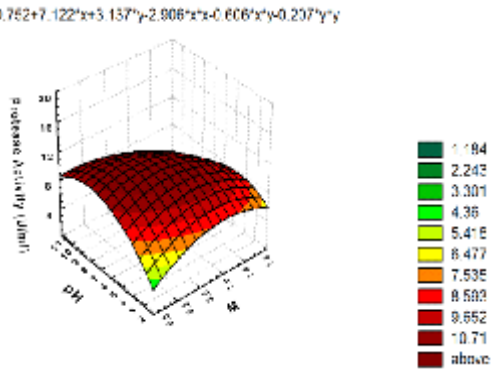

Fig. 2. Response surface plot showing the effect of pH and micronutrient salt solutions concentration on the production of alkaline protease activity by *Bacillus licheniformis*

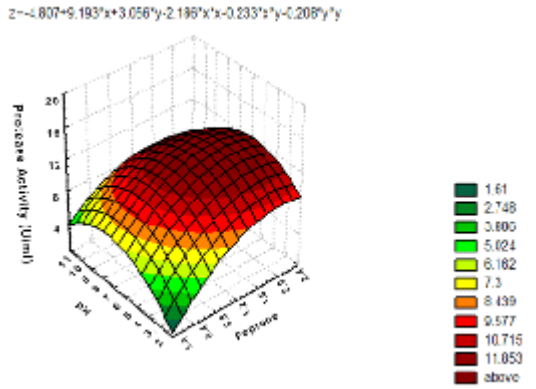

Fig. 3. Response surface plot showing the effect of pH and peptone concentration on the production of alkaline protease activity by *Bacillus licheniformis*

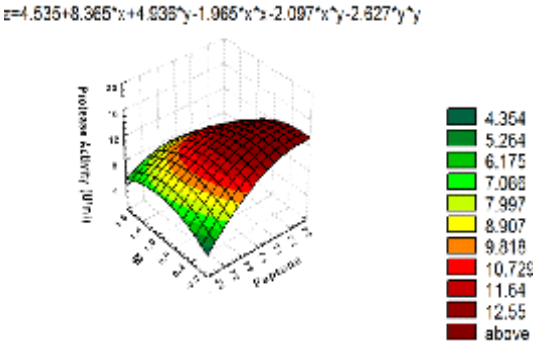

Fig. 4. Response surface plot showing the effect of micronutrient salt solution and peptone concentration on the production of alkaline protease activity by *Bacillus licheniformis*

Table 2. Central composite design by Response surface methodology for independent variables according to experimental plan

Run	Block	X_1 C	X_2 N	X_3 P	X_4 M	X_5pH	X_6 Temp
1	1	0	0	0	0	0	0
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	0	0	0	0	0	0
6	1	0	0	0	-2.378	0	0
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	0	0	0	2.378	0	0
13	1	0	0	2.378	0	0	0
14	1	0	0	0	0	0	0
15	1	0	0	0	0	2.378	0
16	1	1	-1	-1	1	-1	-1
17	1	-1	1	1	-1	1	1
18	1	1	-1	-1	-1	1	-1
19	1	0	0	0	0	0	-2.378
20	1	0	0	0	0	0	0
21	1	1	1	1	-1	1	-1
22	1	1	-1	-1	1	1	1
23	1	-1	1	-1	-1	-1	1
24	1	1	-1	1	-1	1	1
25	1	1	-1	1	1	1	-11
26	1	1	1	1	1	-1	1
27	1	-1	1	1	-1	-1	-1
28	1	0	0	0	0	0	0
29	1	-1	-1	-1	1	-1	1
30	1	0	0	0	0	0	0
31	1	-2.378	0	0	0	0	0
32	1	0	0	-2.378	0	0	0
33	1	-1	-1	-1	-1	-1	-1
34	1	-1	-1	1	1	1	1
35	1	-1	-1	1	-1	-1	1
36	1	0	0	0	0	0	2.378
37	1	1	1	1	1	1	1
38	1	0	-2.378	0	0	0	0
39	1	2.378	0	0	0	0	0
40	1	-1	1	-1	-1	1	-1
41	1	-1	1	1	1	-1	1
42	1	0	2.378	0	0	0	0
43	1	-1	-1	-1	1	1	-1
44	1	0	0	0	0	0	0
45	1	-1	-1	1	1	-1	-1
46	1	-1	1	1	1	1	-1
47	1	1	1	-1	1	1	-1
48	1	0	0	0	0	-2.378	0
49	1	1	1	-1	-1	-1	-1
50	1	-1	-1	-1	-1	1	1
51	1	1	1	1	1	-1	-1
52	1	0	0	0	0	0	0
53	1	0	0	0	0	0	0

Table 3. The minimum and maximum concentrations of selected factors

Run	X_1 C	X_2 N	X_3 P	X_4 M	X_5 pH	X_6Temp
1	1.25	1	1.5	0.55		37
2	2	1.5	2	0.1	5	42
3	2	0.5	2	0.1	5	27
4	2	0.5	1	0.1	5	42
5	1.25	1	1.5	0.55	6.5	37
6	1.25	1	1.5	0.52	6.5	37
7	2	1.5	1	0.1	8	42
8	2	0.5	2	1	5	42
9	0.5	0.5	2	0.1	8	27
10	0.5	1.5	1	1	5	27
11	0.5	1.5	1	1	8	42
12	1.25	1	1.5	1.62	6.5	37
13	1.25	1	2.689	0.55	6.5	37
14	1.25	1	1.5	0.55	6.5	37
15	1.25	1	1.5	0.55	10	37
16	2	0.5	1	1	5	27
17	0.5	1.5	2	0.1	8	42
18	2	0.5	1	0.1	8	27
19	1.25	1	1.5	0.55	6.5	16.6
20	1.25	1	1.5	0.55	6.5	37
21	2	1.5	2	0.1	8	27
22	2	0.5	1	1	8	42
23	0.5	1.5	1	0.1	5	42
24	2	0.5	2	0.1	8	42
25	2	0.5	2	1	8	27
26	2	1.5	1	1	5	42
27	0.5	1.5	2	0.1	5	27
28	1.25	1	1.5	0.55	6.5	37
29	0.5	0.5	1	1	5	42
30	1.25	1	1.5	0.55	6.5	37
31	0.533	1	1.5	0.55	6.5	37
32	1.25	1	0.31	0.55	6.5	37
33	0.5	0.5	1	0.1	5	27
34	0.5	0.5	2	1	8	42
35	0.5	0.5	2	0.1	5	42
36	1.25	1	1.5	0.55	6.5	52
37	2	1.5	2	1	8	42
38	1.25	0.189	1.5	0.55	6.5	37
39	3.03	1	1.5	0.55	6.5	37
40	0.5	1.5	1	0.1	8	27
41	0.5	1.5	2	1	5	42
42	1.25	2.189	1.5	0.55	6.5	37
43	0.5	0.5	1	1	8	27
44	1.25	1	1.5	0.55	6.5	37
45	0.5	0.5	2	1	5	27
46	0.5	1.5	2	1	8	27
47	2	1.5	1	1	8	27
48	1.25	1	1.5	0.55	2.93	37
49	2	1.5	1	0.1	5	27
50	0.5	0.5	1	0.1	8	42
51	2	1.5	2	1	5	27
52	1.25	1	1.5	0.55	6.5	37

Table 4. Results of all independent variables producing protease activity according to experimental plan

Run	X_1	X_2	X_3	X_4	X_5	X_6	EA
1	1.25	1	1.5	0.55		37	12.45
2	2	1.5	2	0.1	5	42	15.2
3	2	0.5	2	0.1	5	27	8.45
4	2	0.5	1	0.1	5	42	8.5
5	1.25	1	1.5	0.55	6.5	37	12.45
6	1.25	1	1.5	0.52	6.5	37	11.95
7	2	1.5	1	0.1	8	42	14.95
8	2	0.5	2	1	5	42	7.1
9	0.5	0.5	2	0.1	8	27	9.8
10	0.5	1.5	1	1	5	27	12.9
11	0.5	1.5	1	1	8	42	15.45
12	1.25	1	1.5	1.62	6.5	37	11.7
13	1.25	1	2.689	0.55	6.5	37	12.95
14	1.25	1	1.5	0.55	6.5	37	12.45
15	1.25	1	1.5	0.55	10	37	11.45
16	2	0.5	1	1	5	27	7.55
17	0.5	1.5	2	0.1	8	42	14.35
18	2	0.5	1	0.1	8	27	1.65
19	1.25	1	1.5	0.55	6.5	16.6	10.15
20	1.25	1	1.5	0.55	6.5	37	12.45
21	2	1.5	2	0.1	8	27	9.3
22	2	0.5	1	1	8	42	12.6
23	0.5	1.5	1	0.1	5	42	17.3
24	2	0.5	2	0.1	8	42	12.8
25	2	0.5	2	1	8	27	8.4
26	2	1.5	1	1	5	42	13.95
27	0.5	1.5	2	0.1	5	27	10.3
28	1.25	1	1.5	0.55	6.5	37	12.45
29	0.5	0.5	1	1	5	42	8.25
30	1.25	1	1.5	0.55	6.5	37	12.45
31	0.533	1	1.5	0.55	6.5	37	11.65
32	1.25	1	0.31	0.55	6.5	37	15.55
33	0.5	0.5	1	0.1	5	27	10.15
34	0.5	0.5	2	1	8	42	9.75
35	0.5	0.5	2	0.1	5	42	7.4
36	1.25	1	1.5	0.55	6.5	52	12.8
37	2	1.5	2	1	8	42	16.7
38	1.25	0.189	1.5	0.55	6.5	37	6.75
39	3.03	1	1.5	0.55	6.5	37	13.55
40	0.5	1.5	1	0.1	8	27	14.8
41	0.5	1.5	2	1	5	42	10.15
42	1.25	2.189	1.5	0.55	6.5	37	16.35
43	0.5	0.5	1	1	8	27	8.15
44	1.25	1	1.5	0.55	6.5	37	12.45
45	0.5	0.5	2	1	5	27	10.45
46	0.5	1.5	2	1	8	27	4.7
47	2	1.5	1	1	8	27	1.3
48	1.25	1	1.5	0.55	2.93	37	10.25
49	2	1.5	1	0.1	5	27	1.3
50	0.5	0.5	1	0.1	8	42	9.95
51	2	1.5	2	1	5	27	10.8
52	1.25	1	1.5	0.55	6.5	37	12.45
53	1.25	1	1.5	0.55	6.5	37	12.45

is the significant of the corresponding coefficient.

After regression analysis according to the polynomial quadric equation, taken all the possible 27 interactions between all independent variables the final results shows that X_1X_4(0.005), (0.042), X_3X_3 (0.007), X_1X_3 (0.002), X_1X_4(0.022), X_1X_6 (0.001), X_2X_4 (0.015), X_2X_6 (0.001) and X_4X_5(0.048) gives highly significant effect on *Bacillus licheniformis.*

Response surfaces plots were plotted to understand the interaction of the medium components and the optimum concentration of

Table 5. Summary data of protease production by *Bacillus* licheniformis

Model		Coefficients(a) Unstandardized Coefficients		Standardized Coefficients	t	Sig.
		B	Std. Error	Beta		
*1	Constant	10.078	13.662		.738	.468
	X_1	-12.866	4.199	-2.428	-3.064	.005*
	X_2	-10.604	6.070	-1.367	-1.747	.094
	X_3	4.251	4.744	.569	.896	.380
	X_4	11.878	5.502	1.330	2.159	.042*
	X_5	2.181	2.323	.873	.939	.357
	X_6	-.272	.331	-.508	-.820	.421
	X_1X_1	.289	.615	.154	.469	.643
	X_2X_2	.199	1.323	.056	.151	.882
	X_3X_3	-.230	.078	-.399	-2.937	.007*
	X_4X_4	-.807	1.706	-.119	-.473	.641
	X_5X_5	-.090	.114	-.472	-.790	.438
	X_6X_6	-.005	.004	-.628	-1.334	.195
	X_1X_2	-.305	1.054	-.080	-.290	.775
	X_1X_3	2.690	.775	.909	3.473	.002*
	X_1X_4	2.735	1.109	.532	2.467	.022*
	X_1X_5	-.372	.364	-.506	-1.022	.318
	X_1X_6	.262	.068	1.862	3.825	.001*
	X_2X_3	-.091	.828	-.024	-.110	.913
	X_2X_4	-4.587	1.740	-.634	-2.637	.015*
	X_2X_5	.012	.548	.011	.021	.983
	X_2X_6	.409	.105	2.060	3.910	.001
	X_3X_4	-.622	1.768	-.120	-.352	.728
	X_3X_5	-.638	.532	-.680	-1.198	.243
	X_3X_6	-.051	.094	-.288	-.543	.592
	X_4X_5	-1.199	.573	-.940	-2.091	.048*
	X_4X_6	-.011	.013	-.126	-.911	.372
	X_5X_6	.032	.035	.589	.915	.370

* Values are significant at 95% confidence limits ($p < 0.05$)

ANOVA(b)

Model		Sum of Squares	df	Mean Square	F	Sig.
1	Regression	506.684	27	18.766	5.105	.000
	Residual	84.543	23	3.676		
	Total	591.227	50			

R=.926, R Square=.857, Adjusted R Square=.689,

each component required for maximum protease production. Response surface plots as a function of two factors at a time, maintaining all other factors at fixed levels (zero, instance), are helpful to understanding the interaction effects of two factors, X and Y. are independent variables and Z consider as dependent variable.

The figure 1 shows the response surface plot that gives significant results indicates that an increases in alkaline protease yield with increase glucose vs pH concentrations, while other variables are maintained at zero level.

Figure 2 shows the response surface plot in which enzyme productivity was observed with the interaction of peptone and pH salt solution on *Bacillus licheniformis.* This interaction shows that the enzyme production was inhibited by higher concentration of these two nutrient sources and more yield observed.

Figure 3 shows the significant effect between the interaction of peptone and micronutrient salt solutions concentration on *Bacillus licheniformis*. Similarly figure 4 shows higher enzyme production observed between interaction of M and pH concentrations. These positive and significant results tell us that the interactions of different factors (sources) enhance the production *Bacillus licheniformis*.

DISCUSSION

Alkaline proteases are most important group of extremoenzymes which have a wide array of applications in various industries such as detergent, food, pharmaceutical, leather and silk[19]. Consequently, the quantity of alkaline proteases produced on a commercial scale worldwide, is greater than any other enzymatic group of biotechnological relevance. Alkaline proteases are generally produced during a fermentation process and secreted into extracellular media as they are produced[4,6,11]. The extracellular proteases are greatly influenced by media components, especially carbon, nitrogen sources and salt solutions and physical factors such as temperature, pH, incubation time, agitation and inoculum density[3,7,13].

The present investigation aimed at optimization of medium components including carbon source (glucose w/v), nitrogen source (peptone w/v), phosphorus source (disodium phosphate w/v), micronutrients (zinc sulfate and magnesium sulfate v/v), pH and temperature which have been suggested to play a significant role in enhancing the production of alkaline proteases by the selected *Bacillus* stains.

Carbon source like glucose is an important carbon source for *Bacillus species*. It has a direct effect on the production of proteases by bacteria as being the main nutritional factor for the Bacillus species. Surabah *et al* (2007) found that protease production increased as the concentrations of glucose increased[20]. It was observed that the production of alkaline protease was enhanced by the addition of protease production *by Bacillus licheniformis*. Nitrogen sources are usually needed for alkaline protease production but requirements of nitrogen sources are different from organism to organism[21]. Nitrogen sources are being backbone nutritional factor for the growth of *Bacillus species* also has a direct effect on the production of proteases. Bhunia *et al* (2010) observed that the organic nitrogen sources are more effective in the production of proteases by *Bacillus licheniformis*. Maximum protease production was observed in culture medium containing peptone as a nitrogen source. Oskouie *et al* (2005) reported that high level of protease production in the presence of peptone as a nitrogen source by *Bacillus* species[22]. The environmental conditions of the fermentation batch play a vital role in the growth and metabolic production of microbial population. The most important among these are the medium pH and incubation temperature[4,11]. Changes in pH and temperature cause denaturation of enzymes, resulting in the loss of catalytic activity. Rehman *et al* (1994) strongly suggested that the requirement of some metal ions play a very essential role for the production of proteases by *Bacillus species*. They found that these metal ions increased the production and stability of proteases[23].

Finally our media optimization results showed that all the nutritional factors impart a directly positive effect for the production of proteases by *Bacillus licheniformis*, which is in agreement with the reported literature.

CONCLUSION

The goal of this study was to optimize

the alkaline protease production from *Bacillus licheniformis* by investigating the effect of environmental conditions during fermentation. Secondly, a media formulation optimized by using response surface methodology was developed for alkaline protease production from strain *Bacillus* strain. Our results concluded that the carbon, nitrogen, pH and temperature have great effect on enzyme production. More studies focusing on large scale production using these conditions are needed to upscale the protease production on industrial level.

ACKNOWLEDGEMENTS

We are thankful to Institute of Molecular Biology & Biotechnology, the University of Lahore, for providing research facilities.

REFERENCES

1. Rao, M.B., Tanksale, A.M., Mohini, S.G., Deshpande, V.V. Molecular and biotechnological aspects of microbial proteases. *Microbiology and Molecular Biology Reviews.,* 1998*;* **62**: 597-5.
2. Adinarayana, K., Ellaiah, P. Response surface optimization of the critical medium components for the production of alkaline protease by a newly isolated *Bacillus sp. Journal of Pharmacy and Pharmaceutical Sciences.,* 2002*;* **5**: 272-8.
3. Mukhtar, H., Ikramul-ul-Haq. Production of alkaline protease by *Bacillus subtilis* and its applications as deplating agents in leather processing. *Pak. J. Bot.,* 2008; **40**:1673 -9.
4. Al-Sehri, M., Abdulrehman, M.Y. Production and some properties of Protease produced by *Bacillus licheniformis* Isolated from Tthmet, Saudi Arabia. *Pak J Biol Sci.,* 2004; **7**:1631-5.
5. Asif, M., Hussain, A., Malik, A.A., Rasool, M. Scale up studies for the production of protease enzyme using *Bacillus subtilis* adopting response surface methodology. *African Journal of Microbiology Research.,* 2012; **6**(9):2120-8.
6. Gupta, R., Beg, Q.K., Lorenz, P. Bacterial alkaline proteases: molecular approaches and industrial applications. *Applied Microbiology and Biotechnology*., 2002b; **59**:15-1.
7. Ikram-ul-Haq., Mukhtar, H., Umber, H. Production of protease by Pencillium chrysogenum through optimization of environmental conditions. *Journal of Agriculture and Social Sciences*., 2006; **2**: 23-5.
8. Anwar, A., Saleemuddin, M. Alkaline protease *from Spilosoma obliqua:* potential applications in bio-formulations. *Biotechnol. Appl. Biochem.,* 2000; **31**: 85-9.
9. Babu, SI., Srinu, B.G.,Kumar, K.N., Kumari, S.K., Yugandhar, M.N., Raju, I. Response surface optimization of critical medium components for the production of alkaline protease by *Asprgillus foetidus*. *Int. J. Chem. Sci.,* 2005; **4**: 951-8.
10. Hajji, M., Rebai, A., Gharsallah, N., Nasri, M. Optimization of alkaline protease production by Asprgillus clavatus ES1 in *Mirabilis jalabilis* jalap tube power using statistical experimental design. *Appl Microbiol. Biotechnol.,* 2008; **76**: 915-3.
11. Nikerel, I.E., Ates, O., Oner, E.T. Effect of bioprocess conditions on growth and alkaline protease production by Halotoleratn *Bacillus lichenifromis* BA17. *Applied Biochemistry and Microbiology*., 2008; **44**: 487-2.
12. Bhunia, B., Dutta, D., Chaudhri, S. Selection of suitable carbon, nitrogen and sulphate source for the production of alkaline protease by *Bacillus licheniformis NCIM-2042*. *Not Sci Bio.,* 2010; **2**(2): 56-9.
13. Gupta, A.V.N., Emmanuel, S., Lakshminaras. Protease production by newly isolated *Bacillus Sp.* Statistical media optimization. *Archives of Applied Science Research.,* 2010; **2**(2): 109-3.
14. Puri, S., Beg, Q.K., Gupta, R. Optimization of alkaline protease from *Bacillus sp*. by response surface methodology. *Current Microbiology.,* 2002; **44**: 286-90.
15. Varun, B, Jones, T.R., Kandasmy, S.KJ., Vijaykumar, P., Anat, A. Optimization of prodciton of subtilisin in solid substrate fermentation using response surface methodology. *Afr. J. Biotech*., 2008; **7**: 2286-1.
16. Lin, S.S., Wen-fang, D., Hong, X., Hua-zhong, L., Zheng-Hong, X., Yan-he, M. Optimization of medium composition for the production of alkaline β-mannase by alkaliphilic *Bacillus sp*. N16-5 using response surface methodology. *Appl Microbiol Biotechnol*., 2007; **75**: 1015-2.
17. Nadeem, M., Qazi, J., Baig, S., Syed, Q. Effect of medium composition on commercially important alkaline protease production by *Bacillus lichenifromis* N-2. *Food Technol. Biotechnol*., 2008; **46**: 388-4.
18. Kunitz, M. Crystalline soybean trypsin inhibitor. *Gene Physiol*., 1947; **30**: 291-10.
19. Prasnthi, V., Yugndhar, M., Nikhu, S.P., Vauddaraju, K., Kumar, N. Optimization of the fermentation media using statistical approach and artificial neural networks for the production

of an alkaline protease from *Bacillus subtilis. Int. J. Nat. Engin. Sc.*, 2008; **2**: 51-6.

20. Saurabh, J., Israr, S.K. A purified method for the detection of microbial proteases on agar plates using Taconic acid. *Biochemistry.*, 2007; **70**: 697-9.
21. Kumar, C.G., Takagi, H. Microbial alkaline proteases: from a bioindustrial viewpoint. *Biotechnology Advances*., 1999; **17**: 561-4.
22. Oskouie, S.F.G.; Tabandeh, F., Yakhchali, B., Eftekhar, F. Enhancement of alkaline protease production by *Bacillus Clausii* using Tagchi experimental design. *African Journal of Microbiology Research.*, 2007; **6**(22): 2559-4.
23. Rahman, R.; Razak, C.N.; Ampon, K.; Basri, M.; Yunus, W.; Salleh, A., Purification and characterization of a heat-stable alkaline protease from *Bacillus stearothermophilus* F1. *Applied Microbiology and Biotechnology.* 1994; **40**: 822-30.