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Full Length Research Paper

Scale up studies for the production of protease enzyme using *Bacillus subtilis* adopting response surface methodology

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We adopted the technique of response surface methodology (RSM) for maximum alkaline protease production by *Bacillus subtilis* during the fermentation process. In RSM, Central Composite Design (CCD) was chosen involving 2^5 full factorial half fractions which were based on 32 independent experiments using various combinations of five nutrient sources at one time. The nutrients provided in the medium were, glucose as carbon source, peptone as nitrogen source, potassium iodine as K source, sodium phosphate as P source, while zinc sulfate and magnesium sulfate were included for provision of micronutrients. The data collected was analyzed through computer-based statistics software 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 value (<0.05).

Key words: Alkaline protease, nutrient, fermentation, response surface methodology, multiple linear regressions.

INTRODUCTION

Proteolytic enzymes are used in a wide range of industrial processes such as food, weave, leather and silk processing, pharmaceutics and detergents production, protein and silver recovery using x-ray films and for the treatment of household wastes. There are also other applications for medical purposes (Adinarayana and Ellaiah, 2002; Al-Sehri and Abdulrehman, 2004; Nadeem et al., 2008; Guangrong et al., 2008; Gupta et al., 2002b). That is why proteolytic enzymes are the most significant group of industrial enzymes which are produced for commercial purposes. Among these enzymes, proteases count for 60% of the world enzyme market in which 25% is made of alkaline proteases and 35% by detergents (Prasnthi et al., 2008; Rao et al., 1998).

Microorganisms are the main and essential source-of enzyme production. *Bacillus* species are an important group which is used to produce alkaline proteases on a large scale, while other important sources include animals and plants (Varun et al., 2008; Babu et al., 2005). The convenience of *Bacillus* strains is due to the fact that they produce large amounts of enzymes in a very short period of time into the fermentation broth. All microorganism including *Bacillus* strains needs its own optimum environmental or physical conditions for maximum growth and enzyme production (Adinarayana and Ellaiah, 2002; Nikerel et al., 2008).

From all of the alkaliphilic bacteria that have been screened in various industrial applications, *Bacillus subtilis* and *Bacillus licheniformis* are found to be the major source of alkaline proteases (Kumar and Takagi, 1999).

Proteases have been classified on the basis of their acid/base behavior into three groups: i.e. acidic, neutral and alkaline proteases. The acidic proteases are those which have their pH optimum in the range of 2.0 to 5.0

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and have mainly fungal origin. The proteases having their pH optimum at 7.1 or around are called neutral. Finally, the third group of proteases that have their pH optimum in the range of 8 to 11 are called alkaline proteases (Mukhtar and Ikramul-ul-Haq, 2008).

The purification process of extracellular protease enzyme production depends on the components of the growth medium (carbon, nitrogen and salt solution), environmental, physical and fermentation factors such as, pH and incubation period, temperature, agitation speed and inoculum density (Hajji et al., 2008; Puri et al., 2002).

The monofactorial method change of one parameter at a time) for optimizing fermentation conditions is most frequently used in microbiology and biotechnology in order to obtain higher yield of the desired product. Not only the method is costly and time consuming than large number of parameters to be considered simultaneously but also ignores the combined interactions of the examined variables (Adinarayana and Ellaiah, 2002; Prasnthi et al., 2008). On the other hand, mathematical and statistical approaches such as RSM and regression analysis are frequently exploited to develop and improve optimization conditions and design experiments to be planned.

The evaluation of factors and the development of models for fermentation and food manufacturing processes are also tributary to those approaches (Gupta et al., 2002b; Nikerel et al., 2008; Lin et al., 2007).

The aim of this study is to improve the production of alkaline proteases by using *Bacillus subtilis*. The optimized medium component sources were carbon, nitrogen, phosphorus, potassium, micronutrients ($ZnSO_4$ and $MgSO_4$) and salt solutions which play a significant role in enhancing the production of alkaline protease (Adinarayana and Ellaiah, 2002; Babu et al., 2005; Puri et al., 2002; Lin et al., 2007; Hanlon et al., 1982). The use of experimentally designed response surface methodology i.e. Central Composite Design (CCD) was successfully applied in this study. Here, we designed and performed 32 experiments using CCD model having two factorials (half fraction) with five factors, cube points 16 and center points in cube 6 along with 10 axial points.

MATERIALS AND METHODS

Bacterial strains

Protease producing strain *B. subtilis* was obtained from Pakistan Type Culture Collection (PTCC) of Food Biotechnology and Research Centre PCSIR Lahore, Pakistan. The strain was maintained on nutrient agar medium for 24 h at 37°C and the cultures were stored and maintained at 4°C.

Detection, isolation and screening of protease enzyme

For detection of alkaline protease producing strains, a detection media (skimmed milk 10 g agar agar 10 g 0.2 M Phosphate buffer pH 7.0) was prepared. The bacterial strains were spread on the detection medium and incubated for 48 h at 37ºC. After the incubation, plates were flooded with 10% tannic acid. The appearance of hollow zones confirmed the digestion of proteins by protease producing colonies, grown over the medium (Saurabh et al., 2007).

Fermentation methodology

Shake flask experiment or cultivation of growth medium

A volume of 100 ml of the growth medium containing glucose, peptone, Na_2P0_4, potassium iodide, $ZnSO_4$ and $MgS0_4$ with different concentrations according to designed experiments were used. The pH was adjusted at 8 for *B. subtilis* (by adding either 1N NaOH or 1N HCl). One percent (by volume) of the inocolum was grown over night (10^8 to 10^{10} cells/ml). Resulting cells were used for proteases production. Cultures were incubated for 24 h at 40°C for *B. subtilis* at 120 rpm on a shaker.

Media optimization

To optimize media components, the following different factors were studied: carbon source, nitrogen source, potassium source, micro-nutrients. The maximum and minimum values of selected factors and their sources were shown in Table 1.

Protease assay to measure the enzymatic activity

The enzymatic activity was measured according to Kunitz (1947) method (Kunitz, 1947). Firstly, phosphate buffer solution (K_2HPo_4 1.74 g/ 100 ml and KH_2Po_4 1.36 g/ 100 ml) was prepared in distilled water and then 1% casein was added. The pH was adjusted around 7. Then, 10% solution of TCA was prepared in distilled water. In all the test tubes, 2 ml of the casein solution and then 1 ml of enzyme solution were added and incubated at 30ºC for 20 min. After incubation 3 ml of 10% TCA solution was added in all test tubes. All the test tubes were centrifuged for 10 min at 5000 rpm and supernatants were used to measure the enzymatic activity at spectrophotometer at 280 nm. One unit of protease was equivalent to the amount of enzyme required to release 1 µg of tyrosine/ml/min under assay conditions.

Experimental design and optimization by RSM

We design our experiments through Response surface methodology using CCD. Central composite experimental design adopted for the optimization and improving total protease production of *Bacillus subtilis*. Two levels factorial with half fraction included 5 factors 2^5, cube points 16 with center points in cube 6, axial points 10, center points in axial 0 with alpha: 2 leading to a total number of 32 experiments was employed. Carbon concentration w/v (X_1 g/L), potassium concentration w/v (X_2 g/L), phosphorus concentration w/v (X_3 g/L), nitrogen concentration w/v (X_4 g/L) and Micronutrients salt solutions v/v (X_5 ml/L) selected as independent variables and dependent variable (Y_1) was *B. subtilis*. The maximum and minimum range of independent variables investigated and full experimental plan with respect to their actual values and coded forms were listed in Tables 2 and 3.

A second degree of quadric polynomial equation was 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 was measured to the independent variables of the experiment.

Table 1. The minimum and maximum values of selected variables.

S/n	Name of Factors	Sources	Max and min range/Conc.
1	Carbon	Glucose	1-5 g/100ml
2	Nitrogen	Peptone	0-4 g/100ml
3	Potassium	Potassium iodide	0.5-4.25 g/100ml
4	Phosphorus	Sodium phosphate	0.5-4.25 g/100ml
5	Micronutrients	$ZnSO_4$ and $MgSO_4$	0.1-1 ul/100ml

Table 2. RSM Central composite design with two factorial half fraction 2^5 factors with coded level values.

Run order	Blocks	Ptt Type	Glucose	K	P	N	Mi
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	-1	0	0	0	-2	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	-2	0	0	0	0
12	1	-1	0	0	0	2	0
13	1	-1	0	0	2	0	0
14	1	0	0	0	0	0	0
15	1	-1	0	0	0	0	-2
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	-1	0	0	0	0	2
20	1	0	0	0	0	0	0
21	1	1	1	1	1	1	1
22	1	-1	-2	0	0	0	0
23	1	1	1	-1	-1	-1	-1
24	1	1	1	-1	1	1	-1
25	1	-1	0	-2	0	0	0
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	-1	0	2	0	0	0
32	1	-1	0	0	-2	0	0

$$Y= \beta_0+ \beta_1X_1+ \beta_2X_2+ \beta_3X_3 + \beta_3X_3++ \beta_4X_4+ \beta_5X_5 +\beta_{11}X^2_1 +\beta_{22} X^2_2+ \beta_{33}+ X^2_3+ \beta_{44} X^2_4+ \beta_{55} X^2_5 +\beta_{12}X_1X_2+ \beta_{13}X_1X_3+ \beta_{14}X_1X_4+ \beta_{15}X_1X_5+ \beta_{23}X_2X_3+ \beta_{24}X_2X_4+ \beta_{25}X_2X_5+ \beta_{34}X_3X_4+ \beta_{35}X_3X_5+ \beta_{45}X_4X_5$$

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

Using the above model to obtain the optimum concentration of the medium components we designed our experimental model in Minitab 15 and SPSS 16 statistical software used for data analysis. To checked the effect of maximum and minimum concentrations C, K, P, N and Micronutrients salt solution of independent variables on

Table 3. Central composite design by Response surface methodology for independent variables in the experimental plan with their actual values.

Run order	X_1 Glucose	X_2 K	X_3 P	X_4 N	X_5 Mi
1	3	1.75	1.75	2	0.55
2	1	0.5	3	1	0.1
3	1	3	0.5	1	0.1
4	1	0.5	0.5	1	1
5	3	1.75	1.75	2	0.55
6	3	1.75	1.75	0	0.55
7	5	3	0.5	3	0.1
8	5	0.5	0.5	3	1
9	1	0.5	3	3	1
10	5	3	3	1	0.1
11	7	1.75	1.75	2	0.55
12	3	1.75	1.75	4	0.55
13	3	1.75	4.25	2	0.55
14	3	1.75	1.75	2	0.55
15	3	1.75	1.75	2	-0.35
16	1	3	3	1	1
17	1	3	3	3	0.1
18	1	3	0.5	3	1
19	3	1.75	1.75	2	1.45
20	3	1.75	1.75	2	0.55
21	5	3	3	3	1
22	-1	1.75	1.75	2	0.55
23	5	0.5	0.5	1	0.1
24	5	0.5	3	3	0.1
25	3	-0.75	1.75	2	0.55
26	1	0.5	0.5	3	0.1
27	5	3	0.5	1	1
28	3	1.75	1.75	2	0.55
29	5	0.5	3	1	1
30	3	1.75	1.75	2	0.55
31	3	4.25	1.75	2	0.55
32	3	1.75	-0.75	2	0.55

dependent variable was *B. subtilis* through draw response surface 3D graphs. All response surface graphs draw in STATISCA 5.5 software.

RESULTS

Production of protease enzyme from *B. subtilis* was conducted in liquid state fermentation. Preliminary experiments on protease production from the above strain indicated that the most important factors were the concentrations of nitrogen, carbon, phosphorus, potassium and micronutrients ($ZnSO_4$ and $MgSO_4$).

The results of Central Composite design experiments studying the effects of five independent variables, viz., C, N, P, K, and micronutrients, concentrations on protease production was presented in Table 4.

Statistical testing of the model was achieved by the Fischer's statistical test for analysis of variance (ANOVA). The model was evaluated by using multiple linear regression analysis and regression coefficients indicated the effect of various factors on the yield of *B. subtilis*. The p-values were used as a tool in order to check the significance of each coefficient, which also indicated the interaction strength between each independent variable. The smaller the p-values, the bigger the significance of the corresponding coefficient. Protease production was evaluated by using a multiple linear regression analysis employing the statistical package for the social sciences software (SPSS 16). Retention of the significant coefficient terms was based on p values (<0.05).

When applying multiple linear regression analysis according to the polynomial quadric equation all

Table 4. Title effect of various factors on enzymatic activity.

PtType	Blocks	Glucose	K	P	N	Mi	EAS U/ml
0	1	3	1.75	1.75	2	0.55	13
1	1	1	0.5	3	1	0.10	12.95
1	1	1	3	0.5	1	0.10	8
1	1	1	0.5	0.5	1	1	10.25
0	1	3	1.75	1.75	2	0.55	14.55
-1	1	3	1.75	1.75	0	0.55	8
1	1	5	3	0.5	3	0.1	3
1	1	5	0.5	0.5	3	1	6
1	1	1	0.5	3	3	1	11.5
1	1	5	3	3	1	0.1	17.5
-1	1	7	1.75	1.75	2	0.55	18
-1	1	3	1.75	1.75	4	0.55	0.6
-1	1	3	1.75	4.25	2	0.55	1
0	1	3	1.75	1.75	2	0.55	2.5
-1	1	3	1.75	1.75	2	-0.35	3
1	1	1	3	3	1	1	6
1	1	1	3	3	3	0.10	8
1	1	1	3	0.5	3	1	6.5
-1	1	3	1.75	1.75	2	1.45	6.25
0	1	3	1.75	1.75	2	0.55	11.75
1	1	5	3	3	3	1	1.6
-1	1	-1	1.75	1.75	2	0.55	11.5
1	1	5	0.5	0.5	1	0.10	15.1
1	1	5	0.5	3	3	0.10	16
-1	1	3	-0.75	1.75	2	0.55	16
1	1	1	0.5	0.5	3	0.10	17.5
1	1	5	3	0.5	1	1	1
0	1	3	1.75	1.75	2	0.55	10.8
1	1	5	0.5	3	1	1	7.8
0	1	3	1.75	1.75	2	0.55	9.8
-1	1	3	4.25	1.75	2	0.55	6.25
-1	1	3	1.75	-0.75	2	0.55	13.25

independent variables of the final results showed insignificant values (Table 5).

The three-dimensional (3-D) response surfaces plots were also helpful to understand the interaction of the medium components and the optimum concentration of 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 were helpful to understanding the interaction effects of two factors. X and Y were independent variables and Z was considered as dependent variable.

The Figure 1 showed significant interaction of N and P on *B. subtilis*, The Figure 2 in which effect of interaction of N and Mi on *B. subtilis* displayed highly significant effect, whereas Figure 3 showed significant effect between the interaction concentrations of P and Mi on *B. subtilis*. These results interprets the impact and interactions of different factors (sources) enhanced the alkaline proteases production of *B. subtilis.*

DISCUSSION

Proteases make up 60-65% of the worldwide industrial enzyme market, in which alkaline proteases contribute a major and important role. Most of the applications in the food industry, peptide synthesis for infant formula, baking and brewing are tributary of alkaline proteases activity. Furthermore, they are also widely used in pharmaceuticals and medical diagnosis. In addition, they intervene in the detergent industry as well as in textile industry in the process of dehairing and leather

Table 5. Applying cond order model of RSM applying multiple linear regressions on *Bacillus subtilis.*

ANOVA					
	Sum of Squares	**Df**	**Mean Square**	**F**	**Sig.**
Regression	579.069	20	28.953	1.071	0.470(a)
Residual	297.489	11	27.044		
Total	876.558	31			

Coefficients					
	Un-standardized Coefficients		**Standardized coefficients**	**t**	***P***
	B	**Std. error**	**Beta**		
(Constant)	9.762	11.017		0.886	0.395
Glucose	-0.841	2.517	-0.278	-0.334	0.745
K	-2.806	3.981	-0.580	-0.705	0.496
P	-0.725	3.981	-0.150	-0.182	0.859
N	7.455	5.370	1.234	1.388	0.192
Mi	5.388	10.847	0.401	0.497	0.629
KP	0.608	0.832	0.337	0.731	0.480
KN	-0.915	1.040	-0.487	-0.880	0.398
PN	-0.290	1.040	-0.154	-0.279	0.786
KMi	0.511	2.311	0.097	0.221	0.829
PMi	-0.856	2.311	-0.162	-0.370	0.718
NMi	1.333	2.889	0.246	0.462	0.653
KG	0.048	0.520	0.043	0.091	0.929
PG	0.540	0.520	0.493	1.038	0.321
NG	-0.659	0.650	-0.574	-1.014	0.332
MiG	-1.597	1.445	-0.500	-1.106	0.292
G2	0.336	0.240	0.712	1.400	0.189
K2	0.280	0.615	0.219	0.456	0.657
P2	-0.360	0.615	-0.280	-0.585	0.570
N2	-1.268	0.960	-0.872	-1.321	0.213
Mi2	-5.861	4.742	-0.528	-1.236	0.242
K	-2.110	2.824	-0.436	-0.747	0.466
N	7.339	4.115	1.214	1.783	0.094
Mi	7.315	8.257	0.545	0.886	0.389
KP	0.490	0.630	0.271	0.778	0.448
KN	-0.915	0.876	-0.487	-1.045	0.312
PMi	-1.142	1.798	-0.216	-0.635	0.534
NMi	1.333	2.433	0.246	0.548	0.591
PG	0.410	0.366	0.374	1.118	0.280
NG	-0.751	0.451	-0.653	-1.664	0.115
MiG	-1.721	1.141	-0.538	-1.508	0.151
G2	0.295	0.159	0.624	1.855	0.082
K2	0.262	0.516	0.204	0.507	0.619
P2	-0.497	0.398	-0.387	-1.248	0.230
N2	-1.297	0.807	-0.892	-1.608	0.127
Mi2	-6.006	3.984	-0.541	-1.508	0.151
K	-1.206	2.141	-0.249	-0.563	0.581
N	7.449	4.019	1.233	1.854	0.081
Mi	7.436	8.071	0.554	0.921	0.370
KP	0.497	0.615	0.275	0.807	0.431
KN	-0.915	0.856	-0.487	-1.068	0.300
PMi	-1.126	1.758	-0.213	-0.641	0.530
NMi	1.333	2.379	0.246	0.560	0.582

Table 5. Contd.

PG	0.418	0.358	0.382	1.167	0.259
NG	-0.744	0.441	-0.648	-1.688	0.110
MiG	-1.712	1.116	-0.536	-1.535	0.143
G2	0.289	0.155	0.612	1.865	0.080
P2	-0.511	0.388	-0.398	-1.315	0.206

R=0.698, R^2=0.487 and Adjusted R^2=0.447.

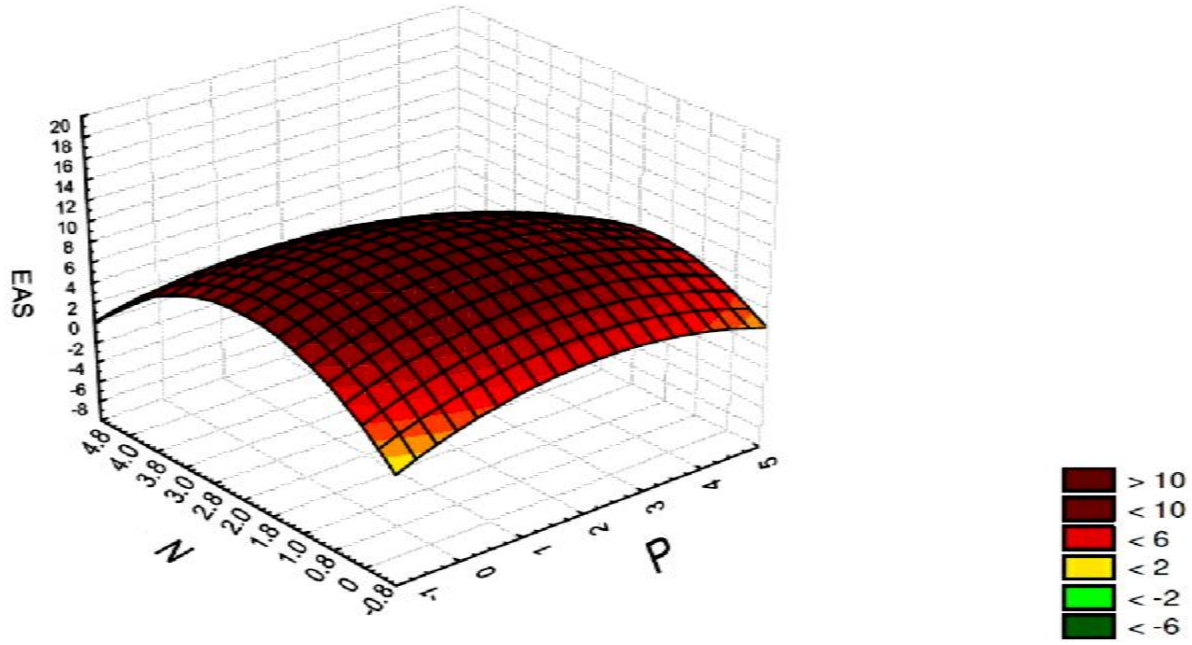

Figure 1. Response surface plot showing the effect on N concentration vs. P concentration on the production of alkaline protease activity of Stain1 *Bacillus subtilis*. 3D surface Plot of EAS1 against P and 16V*32c.
EAS1 = 5.7651 + 1.584*x + 4.7795*y - 0.3869*x*x - 0.29*x*y - 1.3107*.

processing. Currently a large proportion of the commercially available alkaline proteases is derived from *Bacillus* stains. For the reason for this is because of their higher pH and their temperature stability (Adinarayana and Ellaiah, 2002; Mukhtar and Ikramul-ul-Haq, 2008).

Protease producing ability of the selected stains was tested following the procedure of Rahman et al. (2007). Skimmed milk medium was used to check their proteases producing ability, *B. subtilis* showed positive results. Babu et al. (2006) reported that relationship between Carbon sources, nitrogen sources and Micro nutrient salt solutions played a vital role in alkaline protease production by *Bacillus* species.

Adinarayana and Ellaiah (2002) found that protease production increased as long as the concentration of glucose was increasing. It indicates that glucose is the best carbon source for alkaline production by *Bacillus* species.

Nadeem et al. (2008) and Hanlon et al. (1982) observed that nitrogen sources and carbon sources showed maximum alkaline production. Rehman et al. (1994) strongly suggested that requirements of some metal ions for protease production by *Bacillus* species. They found that these metal ions increased the production and stability of proteases.

Conclusion

The present investigation was aimed at the optimization of medium components including carbon source (glucose), nitrogen source (peptone), potassium source (potassium iodine), phosphorus source (disodium phosphate) and micronutrients (zinc sulfate and magnesium sulfate), by RSM Central Composite Design which have been suggested to play a significant role in enhancing the production of alkaline proteases by the selected *Bacillus* strain.

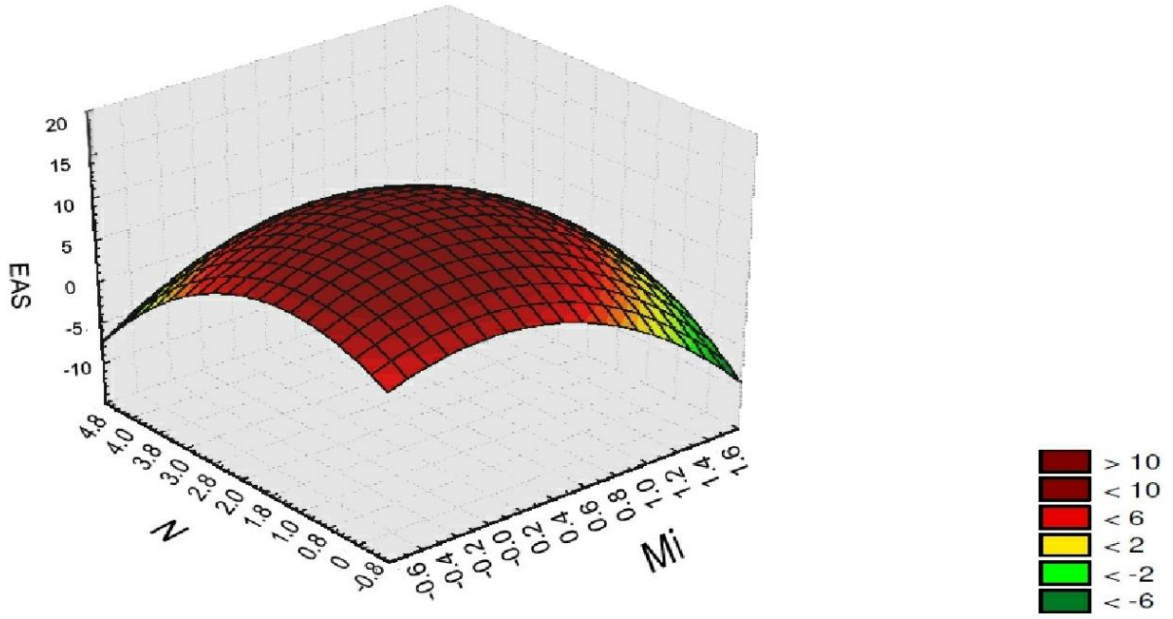

Figure 2. Response surface plot showing the effect on N concentration vs. Mi concentration on the production of alkaline protease activity of Stain1 *Bacillus subtilis*. 3D surface Plot of EAS1 against Mi and N 16v^{w}32c. EAS1=9.3558+0.4674^{w}x+3.7173^{w}y-6.2919^{w}x^{w}+1.3333^{w}x^{w}y-1.3554^{w}y^{w}

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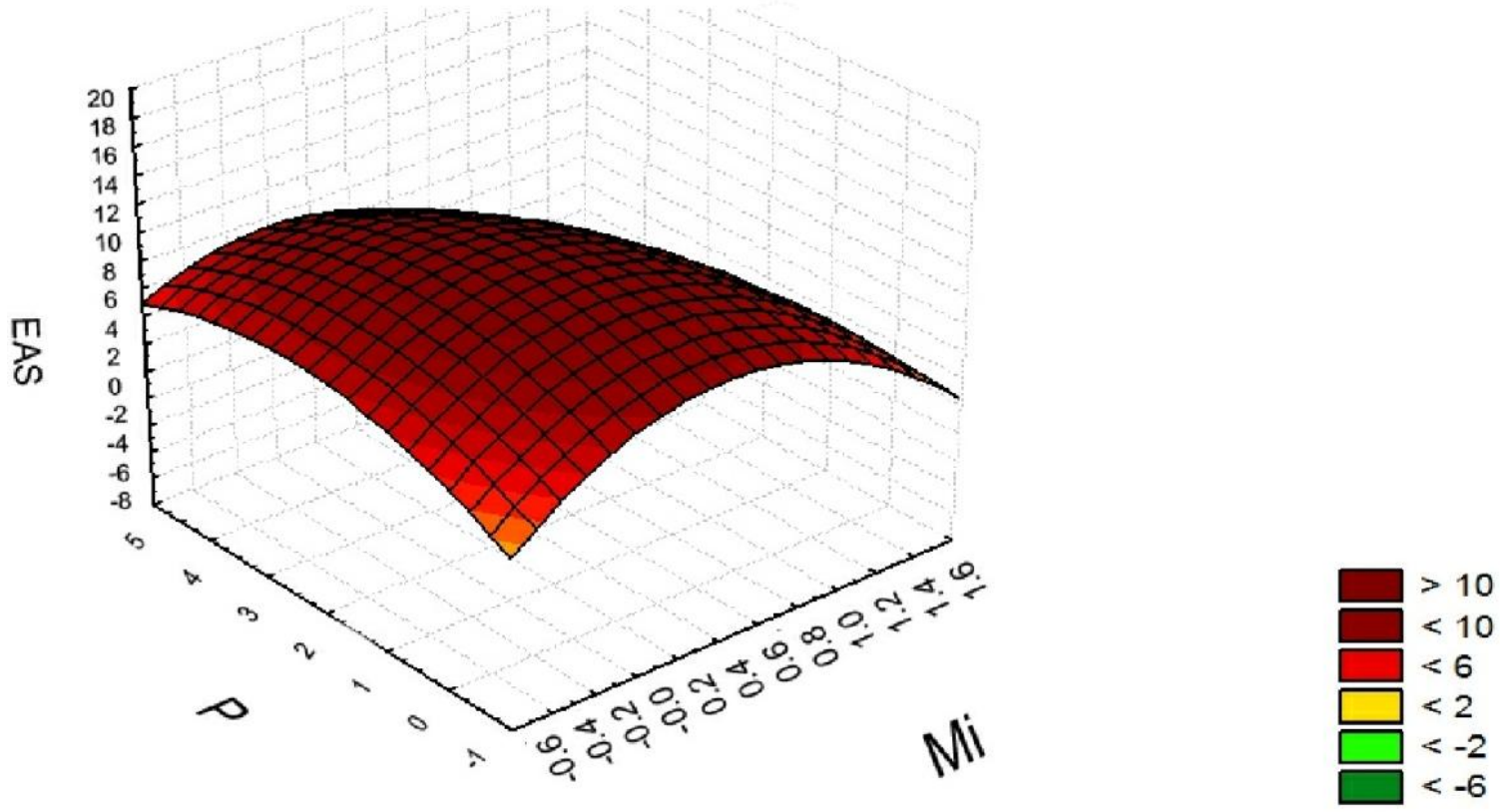

Figure 3. Response surface plot showing the effect on P concentration vs. Mi Concentration on the production of alkaline protease activity of Stain1 *Bacillus subtilis.* 3D surface Plot of EAS1 against Mi and P 16v*32c. EAS1 =9.4545+4.3572*x+1.4616*y-6.0428*x*x-0.8556*x*y-0.3831*y*y.

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