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Full Length Research Paper

Optimization of medium composition for thermostable protease production by *Bacillus* sp. HS08 with a statistical method

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Optimization of the fermentation medium for maximization of thermostable neutral protease production by *Bacillus* sp. HS08 was carried out. After screening various elements, carbon and nitrogen sources, response surface methodology (RSM) was applied to optimize the medium constituents. A 2^4 full-factorial central composite design (CCD) was chosen to explain the combined effects of the four medium constituents; corn starch, soybean meal, glucose and yeast extract. The optimized values obtained by the statistical analysis showed that corn starch at 19.8 g/l, soybean meal at 9.7 g/l, glucose at 3.6 g/l and yeast extract at 3.9 g/l gived maximum protease activity of 6804 U/ml.

Key words: Medium optimization, response surface method, thermostable protease, *Bacillus* sp.

INDRODUCTION

Proteases (EC 3.4) are enzymes that hydrolyze proteins to short peptides or free amino acids. The very importance of enzyme as sources for industrial applications has been well recognized and it was reported that proteases count for nearly 65% of the world enzyme market (Rao et al., 1998). Proteases have a wide range of applications, particularly in food, detergent, weave, leather, pharmaceutical and chemical industries. For example, proteases are widely applied to increase the tenderness of meat. Commercial proteases are mostly produced from various bacteria and it was reported that about 35% of the total microbial enzymes used in detergent industry are the proteases from bacteria sources (Ferrero et al., 1996).

Proteases are commonly classified according to their optimum pH: acidic protease, neutral protease and alkaline protease. There have been extensive researches on the properties and functionalities of acidic or alkaline proteases. One particular interest is the production of alkaline protease from bacillus for applications in detergent industry (Ferrero et al., 1996; Manachini and Fortina, 1998; Johnvesly et al., 2002; Fu et al., 2003). Thermophiles such as *Bacillus stearothermophilus* (Boonyanas et al., 2000), *Thermus aquaticus* (Gabriela et al., 2003), *Bacillus licheniformis* (Ferrero et al., 1996; Nedra El Hadj-Ali et al., 2007), *Bacillus pumilus* (Kumar, 2002), *Geobacillus* (Zhu et al., 2007) and *Thermoanaerobacter yonseiensis* (Hyenung et al., 2002) have been studied for their capability in producing thermostable proteases. Biochemical properties of the enzymes produced from these thermophilies have also been well investigated. Because of their high activity and stability at elevated temperatures, the thermophilic proteases can also be used as ideal models for studying thermal stability of protein (Rao et al., 1998). Among these enzymes, the thermostable proteases have been extensively studied not only for industrial production such as detergent, weave processing, meat tendering and reducing the risk of contamination by other organisms at high temperature, but also for the elucidation of mechanisms involved in thermostability of enzymes (Helmann, 1995; Imanaka et al., 1986).

Given the potential uses of the thermostable proteases and their high demand, the need exists for the discovery of new strains of bacteria that produce enzymes with no-

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vel properties and the development of low cost industrial medium formulations. In this respect, the response surface method, which is used to study the effects of several factors influencing the responses by varying them simultaneously and carrying out a limited number of experiments (Adinarayana and Ellaiah, 2002), was thought to fit to the scope of this study. Optimization of media components by classical methods which involves the change of one variable at a time, was extremely time consuming and expensive when a large number of variables were considered. In order to overcome this difficulty and determine the interaction between the studied variables, an experimental factorial design and response surface methodology (RSM) was employed for the optimization process. It was well documented that media components greatly influence the extracellular production of proteases in microorganisms and their interaction plays an important role in the synthesis of these enzymes (Puri et al., 2002). Therefore, the objective of this study was to optimize the fermentation medium by applying RSM for the production of a thermostable neutral protease with possible novel characteristics.

This enzyme was produced by *Bacillus* sp. HS08, newly isolated from a soil sample collected from Toulufan Crater, Xingjiang Province, China (Huang et al., 2006).

MATERIAL AND METHODS

Microorganism and culture conditions

The *Bacillus* sp. HS08 was first isolated in this laboratory from a soil sample collected from Toulufan Crater, Xingjiang Province, China. The strain was then left to grow in a medium containing 0.5% (w/v) yeast extract and 1.0% (w/v) peptone. For submerged cultivation, 5% (v/v) inocula level was used. The cultivation was performed with 500 ml Erlenmeyer flask containing 200 ml of different composition medium and the culture was allowed to grow at 46°C for 18 h on a rotary shaker (200 rpm).

Determination of protease

The culture solution was centrifuged at 14,000 g for 30 min at a cool temperature of 4°C (Hitach CF15R, Hitachi Koki Co. Ltd., Japan). The supernatant was assayed for proteolytic activity as the following. A mixture of 400 µl casein solution (2% w/v in 50 mM Tris-HCl buffer pH7.2) and 100 µl of the sample were added to a tube. The reactions were carried out at 65°C for 10 min and then terminated by the addition of 1 ml 10% trichloroacetic acid (TCA, w/v). The mixture was centrifugation at 14,000 g for 20 min and a total of 200 µl supernatant was carefully removed to measure tyrosin content using a Folin-phenol method (Michel and Francosi, 1986). One unit of protease activity (U) was defined as the amount of enzyme that hydrolyzed casein to produce 1 µg tyrosin within 1 min at 65°C.

Selection of carbon and nitrogen sources

Various simple and complex carbon (glucose, corn starch, sucrose and maltose) and nitrogen sources (soybean meal, yeast extract, $(NH_4)_2SO_4$ and peptone) were supplemented individually to the cultivation medium. All these sources were added in the production medium at a final concentration of 3 and 1% (w/v) for carbon and nitrogen, respectively. The initial pH of the medium was set at 7, and with no control of pH during the shaker-flask cultivation. The protease yield was determined after 18 h of incubation at 46°C under shaking (200 rpm).

Effect of some elements and surfactant

Different elements ($CaCl_2$, $MgSO_4$, KH_2PO_4, $ZnSO_4$, $CuSO_4$ and $FeSO_4$) and Tween 80 with various concentrations were supplemented individually to the basic medium and incubated at 46°C under shaking (200 rpm).

Central composite design

A central composite design (CCD) was described to approach the interaction among different factors. For a 2^4 factorial CCD with four factors at two levels, 16 experimental runs were required with eight star points (α=2) and two replications of the central points, leading to a total 26 sets at five levels of inocula. The model fitted to the centered data on the response of protease activity was of second-order polynomial function:

$$Y = a_0 + \sum_{i=1}^{n} a_i X_i + \sum_{i=1}^{n-1} \sum_{j=i+1}^{n} a_{ij} X_i X_j \quad (1)$$

where Y is the predicted response, i and j values are from 1 to the number of variables (n), a_0 was the intercept term, a_i values are the linear coefficient, a_{ij} values are the quadratic coefficient and X_i and X_j were the level of the independent variables.

Statistical analysis

Data from CCD were subjected to second-order multiple regression analysis using least-squares regression methodology to obtain the parameters of the mathematical models. Statistica, Version 7.0 (Statsoft Inc., Tulsa, OK) was used for the regression analysis of the experimental data obtained. This software also generated response surface plots.

RESULTS AND DISCUSSION

Effect of carbon and nitrogen sources

In order to study the effect of carbon and nitrogen sources on protease production, cultivations were performed with 3% carbon (glucose, maltose, sucrose and corn starch) and 1% nitrogen (soybean meal, yeast extract, peptone and $(NH_4)_2SO_4$) sources. As shown in Table 1, protease activity varied markedly in a range of 60 – 3180 U/ml. Very low protease activity production was founded when maltose was used as carbon sources or peptone as nitrogen source. Relative low protease activity production was found when an inorganic nitrogen source $(NH_4)_2SO_4$ was applied. The maximal protease activity of 3540 U/ml was found with the combinations of corn starch and soybean meal. This confirms earlier reports, where it has been observed that the complex

Table 1. Effect of nitrogen and carbon sources of the thermostable protease production[a].

Nitrogen source	Carbon source			
	Glucose	Maltose	Corn starch	Sucrose
Soybean meal	2780	365	3540	2540
Yeast extract	2650	287	3180	2330
Peptone	80	75	90	60
$(NH_4)_2SO_4$	890	185	1420	1180

[a]The carbon sources and nitrogen sources were 3 and 1%, respectively.
The unit of protease was U/ml.

Table 2. Effects of some elements in basic medium on the thermostable protease production.

Element	Conc. (g/l)	Relatively activity (%)
Control		**100**
$CaCl_2$	0.1	125
	1.0	87
$MgSO_4$	0.1	118
	1.0	89
KH_2PO_4	0.5	105
	2.0	98
$ZnSO_4$	0.1	106
	0.5	93
$CuSO_4$	0.1	56
	0.5	24
$FeSO_4$	0.1	75
	0.5	48
Tween-80	0	100
	0.3	127

carbon and nitrogen sources were better substrates for protease production than simple sugars, such as glucose, which caused catabolite repression (Kucera, 1971; Calik and Ozdamar, 2001; DeAzeredo et al., 2003). But the simple sugars could be the better growth carbon source for bacteria. Thus, carbon sources of glucose and corn starch, nitrogens sources of soybean meal and yeast extract were selected for the CCD experiment.

Effect of some elements and surfactant

In order to investigate the effects of different elements on protease activity, the reference media were enriched with additions of $MgSO_4$ (0.1 or 1.0 g/l), KH_2PO_4 (0.5 or 2.0 g/l), $CaCl_2$ (0.1 or 1.0 g/l), $ZnSO_4$ (0.1 or 0.5 g/l), $CuSO_4$ (0.1 or 0.5 g/l), $FeSO_4$ (0.1 or 0.5 g/l) and tween 80 (0 or 0.3 g/l). Tween 80 was used in these experiments in order to determine if it had a promoting or inhibiting effect on the enzyme synthesis. Tween 80 is a well known water soluble surfactant, which could facilitate the nutrient and oxygen transfer to the microorganisms in the broth. The decision on the concentrations of these components was based on literature data used for these types of fermentations (Bamerji et al., 1999; Johnvesly and Naik, 2001; Ellouz et al., 2001). $CaCl_2$ and $MgSO_4$ at 0.1 g/l and Tween 80 at 0.3 g/l enhanced enzyme synthesis if used separately (Table 2). These three components resulted in 25, 18 and 27% more enzyme activity, respectively, as compared with the control. The stimulating effect of $CaCl_2$ was also reported by Mabrouk et al. (1999) who contributed this to the stabilizing effect of $CaCl_2$ on the alkaline protease. The higher $CaCl_2$ concentration (1.0 g/l) had an inhibitory effect resulting in an activity of 87% as compared with the control. The presence of $CuSO_4$ and $FeSO_4$ at more than 0.1 g/l would inhibited the thermostable protease synthesis. The other components at the given concentrations did not influence the enzyme synthesis very much.

In order to determine the effect of concentrations of the three compositions on enzyme synthesis, a serial con-centrations of the three compositions were tested in next experiments. The results were shown in Figure 1. From these results, the presence of $CaCl_2$ at 0.2 g/l, $MgSO_4$ at 0.2 g/l and Tween 80 at 0.20 g/l were the best concen-trations.

Optimization by central composite design

In order to search for the optimum combination of complex carbon and nitrogen components of the medium, experiments were performed according to the CCD experimental plan (Tables 3 and 4). The results of the second order response surface model fitting in the form of ANOVA were given in Table 5. The application of RSM yielded the following regression equation which was empirical relationaship between protease activity (*Y*) and the test variables.

$$Y = -15936.0 + 558.7X_1 - 7.5X_1^2 + 730.3X_2 - 25.2X_2^2 + 3478.3X_3 - 398.2X_3^2 + 3714.0X_4 - 379.4X_4^2 - 13.1X_1X_2 - 20.0X_1X_3 - 16.0X_1X_4 + 14.8X_2X_3 - 9.5X_2X_4 - 85.5X_3X_4 \quad (2)$$

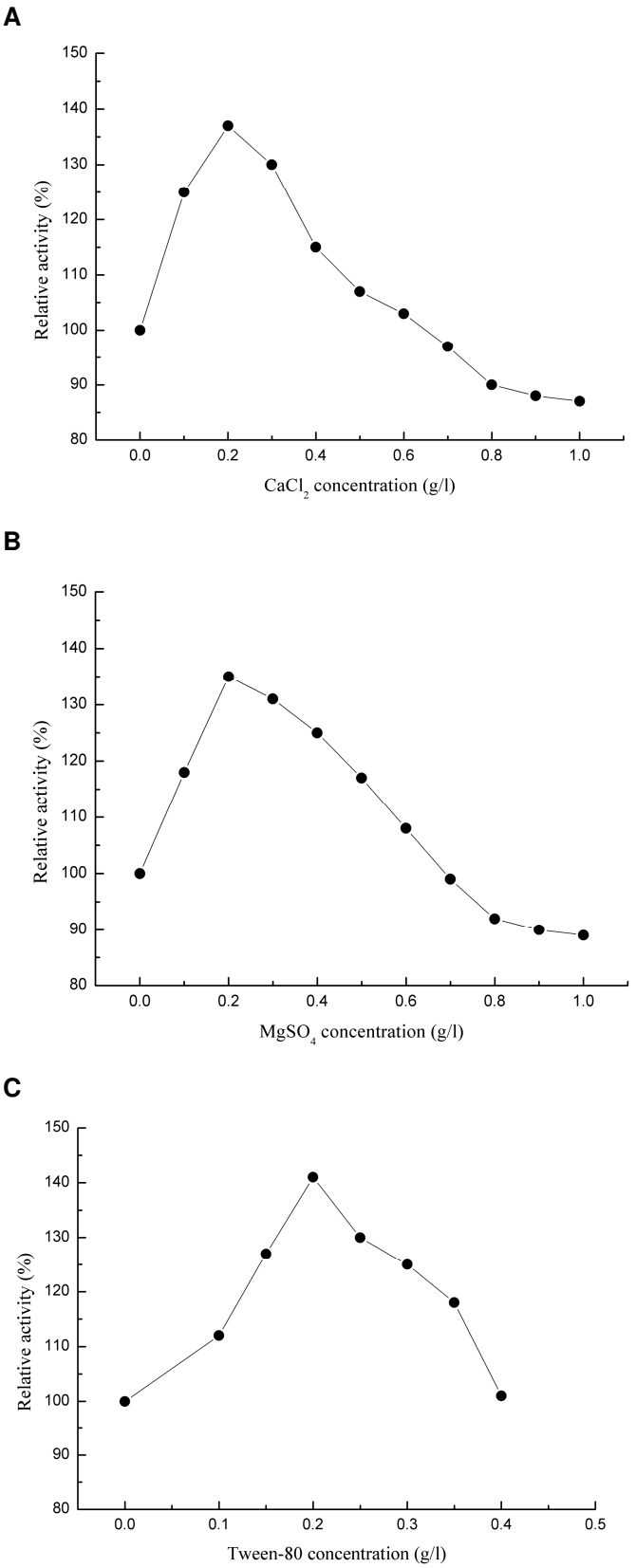

Figure 1. Effect of $CaCl_2$ (A), $MgSO_4$ (B) and Tween 80 (C) concentraction in medium composition on the thermostable protease production by *Bacillus* sp. HS08.

where Y was the response (protease activity) and X_1, X_2, X_3 and X_4 were the variables, corn starch, soybean meal, glucose and yest extract concentration, respectively.

Student t-test of each coeffecient of the model (as shown in Table 5) showed that all the four quardratic terms had siginificant effect ($p<0.01$) on protease production, which the linear terms and most of cross product of any two factors did not. ANOVA indicated that the quadratic models derived from RSM could adequately be used to describe the medium concentrations and the protease yield (Y) under a wide range of operating conditions. The R^2 (multiple correlation coefficient) of the regression equation obtained from ANOVA was 0.835, which meant that the model could explain 83.5% variation in the response. The location of optimum was determined to be X_1 = 19.8, X_2 = 9.7, X_3 = 3.6 and X_4 = 3.9 obtained by the differentiation of the quadratic model given by Eq. (2). The predicted optimal protease activity corresponding to these values was 6754 U/ml. In order to test the reliability of the model in predicting maximum protease activity, three additional experiments using this optimum medium composition were performed. The three replicate experiments yielded an average maximum protease activity of 6804 U/ml. The good agreement between the predicted and experimental results verified the validity of the model and existence of an optimal point.

The 3D response surface and the 2D contour plots were generally the graphical representations of the regression equation; the 2D contour plots are presented in Figure 2 from which the values of protease activity for different concentrations of the variables could be predicted. Each contour curve represented an infinite number of combinations of two test variables with the other two maintained at their respective zero level. The maximum predicted value was indicated by the surface confined in the smallest ellipse in the contour diagram. The contour plots in Figure 2 showed that there were ellipsis near the centre point, which meant there were well defined optimum carbon and nitrogen sources. However the ellipsis was not small enough, as the surfaces were rather symmetric and a little flat near the optimum. Although the actual situation could be more complicated than that we had reported here, an attempt for the medium optimization had been made by RSM.

There had been a number of studies conducted on optimization of different physicochemical parameters of different organisms using response surface methodology (Chauhan and Gupta, 2004; Rahman et al., 2004; Beg et al., 2003; Lui et al., 2004). In general there was no defined medium designed for the production of protease from different microbial sources (Chauhan and Gupta, 2004; Li et al., 2002; Ramirez et al., 2001). Every organism was unique in its requirement for the mentioned parameters for maximum enzyme production. Therefore, each of them had to be considered separately and the requirements had to be optimized accordingly. All these studies (Roberta et al., 2006; Rao et al., 2006; Rodrigues

Table 3. Experimental codes, ranges and levels of the independent variables for response surface methodological experiment.

Variables	Unit	Symbol code	Levels				
			-1.68	-1	0	+1	+1.68
Corn starch	g/l	X_1	3.5	12.0	20.0	28.0	36.5
Soybean meal	g/l	X_2	1.8	6.0	10.0	14.0	18.2
Glucose	g/l	X_3	1.9	3.0	4.0	5.0	6.1
Yeast extract	g/l	X_4	1.9	3.0	4.0	5.0	6.1

Table 4. Central composite design matrix for the experimental design and predicted responses for protease activity.

Run order	Blocks	Experimental values				Protease activity (U/ml)	
		X_1	X_2	X_3	X_4	Observed[a]	Predicted
1	1	12.0	6.0	3.0	3.0	5233	4828
2	1	12.0	6.0	3.0	5.0	4921	5175
3	1	12.0	6.0	5.0	3.0	4096	4598
4	1	12.0	6.0	5.0	5.0	4350	4603
5	1	12.0	14.0	3.0	3.0	5470	5508
6	1	12.0	14.0	3.0	5.0	5236	5703
7	1	12.0	14.0	5.0	3.0	5685	5515
8	1	12.0	14.0	5.0	5.0	5514	5368
9	1	28.0	6.0	3.0	3.0	5896	5982
10	1	28.0	6.0	3.0	5.0	5364	5816
11	1	28.0	6.0	5.0	3.0	5299	5112
12	1	28.0	6.0	5.0	5.0	4703	4604
13	1	28.0	14.0	3.0	3.0	4959	4985
14	1	28.0	14.0	3.0	5.0	5231	4668
15	1	28.0	14.0	5.0	3.0	4669	4352
16	1	28.0	14.0	5.0	5.0	3008	3692
17	1	20.0	10.0	4.0	4.0	6803	6693
18	2	3.5	10.0	4.0	4.0	5142	4928
19	2	36.5	10.0	4.0	4.0	4407	4391
20	2	20.0	1.8	4.0	4.0	5397	5104
21	2	20.0	18.2	4.0	4.0	4787	4866
22	2	20.0	10.0	1.9	4.0	5687	5626
23	2	20.0	10.0	6.1	4.0	4536	4386
24	2	20.0	10.0	4.0	1.9	4937	5247
25	2	20.0	10.0	4.0	6.1	5445	4925
26	2	20.0	10.0	4.0	4.0	6630	6693

[a]The observed values of protease activity were the means of triplicates.

et al., 1998; Lui and Tzeng, 1998; Ramirez et al., 2001)clearly showed that this technique was very powerful in the optimization of bioprocesses. Using cost effective media formulation and optimizing it at its minimum requirements for maximum enzyme production was extremely important in industrial scale for economic reasons. Therefore, using common industrial ingredients such as soybean meal, corn starch and Tween 80, which were known for their low cost and then optimizing the process using response surface methodology would serve as a potential example for the applications used in industrial microbial fermentations.

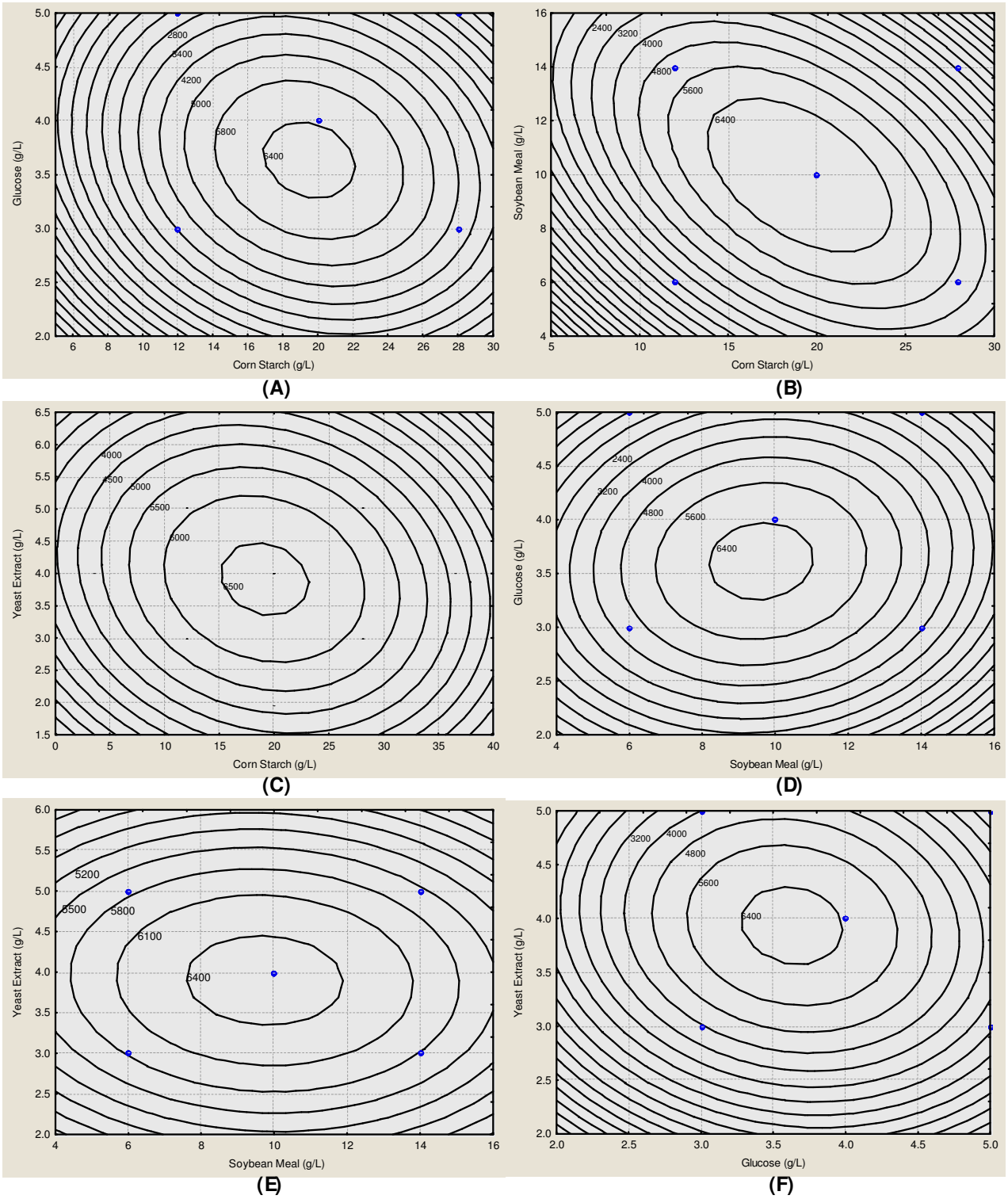

Figure 2. Counter plot of the thermostable protease (U/ml): the effect of corn starch and glucose (A), corn starch and soybean meal (B), corn starch and yeast extract (C), soybean meal and glucose (D), soybean meal and yeast extract (E), glucose and yeast extract (F) on the protease production. Other various were held at zero level.

Table 5. Analysis of variance (ANOVA) for the regression model of protease production obtained from the experimental results.

Source	Sum of squares	Degree of freedom	Mean of squares	F-value	p-value
X_1 (L)	340985	1	340985	1.42397	0.260293
X_1 (Q)	4293185	1	4293185	17.92857	0.001732 [a]
X_2 (L)	73967	1	73967	0.30889	0.590579
X_2 (Q)	3051529	1	3051529	12.74335	0.005098 [a]
X_3 (L)	2210498	1	2210498	9.23116	0.012499
X_3 (Q)	2982162	1	2982162	12.45367	0.005456 [a]
X_4 (L)	152937	1	152937	0.63867	0.442759
X_4 (Q)	2707608	1	2707608	11.30712	0.007211 [a]
X_1 by X_2	2805625	1	2805625	11.71644	0.006515 [a]
X_1 by X_3	408321	1	408321	1.70517	0.220853
X_1 by X_4	263682	1	263682	1.10115	0.318712
X_2 by X_3	55932	1	55932	0.23358	0.639292
X_2 by X_4	23104	1	23104	0.09648	0.762469
X_3 by X_4	116964	1	116964	0.48845	0.500552
Error	2394605	11	239461		
Total SS	14481687	25			

[a]Siginificant at 99% level.

Conclusion

Complexes of carbon and nitrogen sources in the culture medium could improve the production of the thermostable neutral protease from *Bacillus.* sp. HS08. The RSM optimized shaker-flask cultivation medium composition is (in g/l, w/v): 19.9 corn starch, 9.7 soybean meal, 3.6 glucose and 3.9 yeast extract. Applying the RSM medium for the protease production could reach 6804 U/ml in 18 h, which was very close to the RSM model predicted values of 6754 U/ml.

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