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Production of thermostable pullulanase by *Clostridium thermosulfurogenes* SV2 in solid-state fermentation: optimization of enzyme leaching conditions using response surface methodology

P. Rama Mohan Reddy, S. Mrudula, B. Ramesh, Gopal Reddy, G. Seenayya

Abstract The optimization of parameters for the effective leaching of thermostable pullulanase from *Clostridium thermosulfurogenes* SV2-fermented bran was carried out using response surface methodology based on the central composite rotatable design. The design contains a total of 54 experimental trials with the first 32 organized in a fractional factorial design and experimental trials from 33–40 and 51–54 involving the replication of the central points. The design was employed by selecting solvent to wheat bran ratio (S/BB), process temperature, solvent pH, shaking (RPM) and contact time (*h*) as model factors. Among the five independent variables studied, the S/BB, solvent pH and shaking were found to be significant. S/BB ratio of 9.0, 200 RPM shaking and solvent pH 6.0 were identified as optimum for the leaching of thermostable pullulanase from the strain SV2-fermented bran.

1 Introduction

In recent years, pullulanase (pullulan 6-glucanohydrolase, EC 3.2.1.41), a debranching enzyme, has been gaining importance in starch conversion processes [1]. It cleaves α-1,6 linkages in pullulan, amylopectin and other related polysaccharides [2]. The enzyme can be used together with the exo-acting enzymes such as glucoamylase or *β*-amylase, which are not capable of bypassing (hydrolyzing) the α-1,6 glucosidic linkages at the amylopectin branch points or do so at reduced rates [3, 4], to improve the yield of the final starch hydrolysis product [1]. A high value is placed on thermostable and thermoactive enzymes in the bioprocessing of starch, since the bioprocessing of starch at elevated temperature improves the solubility of starch, decreases its viscosity, limits microbial contamination, reduces reaction times and becomes more economical. Thermoanaerobic organisms show promise for the production of thermostable amylolytic and pullulolytic enzymes, and efforts have been made to isolate thermoanaerobic bacteria that produce thermostable pullulanase [5–9]. In this direction, we have isolated anaerobic, thermophilic and amylolytic bacterium, *Clostridium thermosulfurogenes* SV2 that produces high yields of thermostable pullulanase [10]. The enzyme has been purified to homogeneity and characterized [11], and studied its production in submerged [12] and solid-state fermentation [13]. Earlier, we have employed Plackett–Burman design for screening of various nutrients [14] and response surface methodology for the optimization of the levels of selected nutrients [15] for the production of thermostable pullulanase by *C. thermosulfurogenes* SV2 in SSF.

In recent times, the bacterial systems are increasingly investigated for the production of enzymes and metabolites by solid-state fermentation (SSF) [16]. The SSF has numerous advantages over submerged fermentation (SmF), including superior productivity, simple technique, low capital investment, low energy requirement and less water output, better product recovery and lack of foam build-up [17] and reported to be the most appropriate process for developing countries.

Leaching of enzymes from fermented bran is a difficult task, and is an important aspect for the development of cost effective process for enzyme production in SSF. Experimental design techniques are very useful tools for this purpose, as they can provide statistical models which help in understanding the interactions among the process parameters at varying levels and in calculating the optimal level of each parameter for a given target (maximum enzyme leaching) [14, 15, 18]. Another important advantage with the use of statistical models in optimization processes is the requirement of a very less number of experiments and thereby resulting in saving of time, glassware, chemicals and manpower [19, 20]. In spite of the above advantages, the statistical designs are applied to a limited number of fermentation processes [14, 15, 18, 19, 21]. In the present study, the optimization of five selected physical parameters was carried out using response surface methodology for the effective leaching of thermostable pullulanase from *C. thermosulfurogenes* SV2-fermented bran.

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P. Rama Mohan Reddy*, S. Mrudula, B. Ramesh,
Gopal Reddy, G. Seenayya (✉)
Department of Microbiology,
Osmania University,
Hyderabad 500 007, A.P., India

Present address:
* Scientist, Informatics Division,
Monsanto Research Center,
At Indian Institute of Science,
Bangalore 560 012, India

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2
Materials and methods

2.1
Microorganism and culture conditions

The bacterial strain *Clostridium thermosulfurogenes* SV2 employed in the present study was isolated from starch industry wastes [10] using TYE medium [22].

2.2
Solid-state fermentation technique

The solid-state fermentation was carried out anaerobically at 60 °C in 120 ml serum vials that contained a pre-reduced and sterilized optimum medium as described earlier [15]. During incubation, the contents in the vials were periodically mixed by gentle shaking and the accumulated gases were intermittently removed by using a sterile needle. At the end of the incubation, the vials were taken out and the enzymes from each vial were extracted with 0.1 M phosphate buffer (pH 6.0) at a 1:5 (w/v) ratio at room temperature.

The levels of the selected parameters were fixed based on the literature and on our own experience gained.

2.3
Experimental design

The experiments were conducted in a randomized fashion by adopting the central composite rotatable design (CCRD) (Table 1). The CCRD contains a total of 54 experiments with the first 32 experiments organized in a fractional factorial design, with the experimental trials from 33–40 and 51–54 involving the replications of the central points. Once the experiments are performed, the co-efficient of polynomial model is calculated using the equation [23]:

$$Y = b_0 + \sum b_i x_{\mathrm{I}} + \ \leq \sum b_{ij}\, x_i x_j + \sum_{\mathrm{I}} \leq \sum_j \leq \sum_k b_{ijk}\, x_i x_k + e \ ,$$

where, *i*, *j*, *k* are linear, quadratic and cubic co-efficients, respectively, while '*b*' is regression coefficient and '*e*' is random error. The significance of each co-efficient was determined using the student *t*-test [20]. Model terms were selected or rejected based on the student *t*-value [24] or significance. Three-dimensional plots of two factors (at five different levels each) versus the amount of enzyme leached were drawn by keeping the other three factors at their optimum levels. The results were analyzed by using the 'Indostat statistical package'. Three-dimensional plots and their respective contour plots were obtained based on the effect of levels of two parameters and their interactions on the yield of pullulanase by keeping the other three parameters at their optimal concentrations (as obtained through ANOVA). From these three-dimensional plots, the interaction of one parameter with other parameter was studied. The optimum concentration of each parameter was identified based on the hump in the three-dimensional plots or from the central point of the corresponding contour plot.

2.4
Pullulanase assay

The pullulanase activity in the clarified samples was measured by incubating 0.5 ml appropriately diluted

Table 1. The central composite rotatable design for optimization of five paramenters (each at five levels) for the leaching of thermostable β-amylase and pullulanase from *C. thermosulfurogenes* SV2-fermented bran

C.No.*	Code values and real values*					C.No.*	Code values and real values•				
	Factor X_1	Factor X_2	Factor X_3	Factor X_4	Factor X_5		Factor X_1	Factor X_2	Factor X_3	Factor X_4	Factor X_5
1	−1 (4)	−1 (1.0)	−1 (5.0)	−1 (30)	−1 (50)	23	−1 (4)	1 (2.0)	1 (7.0)	−1 (30)	1 (150)
2	1 (8)	−1 (1.0)	−1 (5.0)	−1 (30)	−1 (50)	24	1 (8)	1 (2.0)	1 (7.0)	−1 (30)	1 (150)
3	−1 (4)	1 (2.0)	−1 (5.0)	−1 (30)	−1 (50)	25	−1 (4)	−1 (1.0)	−1 (5.0)	1 (50)	1 (150)
4	1 (8)	1 (2.0)	−1 (5.0)	−1 (30)	−1 (50)	26	1 (8)	−1 (1.0)	−1 (5.0)	1 (50)	1 (150)
5	−1 (4)	−1 (1.0)	1 (7.0)	−1 (30)	−1 (50)	27	−1 (4)	1 (2.0)	−1 (5.0)	1 (50)	1 (150)
6	1 (8)	−1 (1.0)	1 (7.0)	−1 (30)	−1 (50)	28	1 (8)	1 (2.0)	−1 (5.0)	1 (50)	1 (150)
7	−1 (4)	1 (2.0)	1 (7.0)	−1 (30)	−1 (50)	29	−1 (4)	−1 (1.0)	1 (7.0)	1 (50)	1 (150)
8	1 (8)	1 (2.0)	1 (7.0)	−1 (30)	−1 (50)	30	1 (8)	−1 (1.0)	1 (7.0)	1 (50)	1 (150)
9	−1 (4)	−1 (1.0)	−1 (5.0)	1 (50)	−1 (50)	31	−1 (4)	1 (2.0)	1 (7.0)	1 (50)	1 (150)
10	1 (8)	−1 (1.0)	−1 (5.0)	1 (50)	−1 (50)	32	1 (8)	1 (2.0)	1 (7.0)	1 (50)	1 (150)
11	−1 (4)	1 (2.0)	−1 (5.0)	1 (50)	−1 (50)	33–40	0 (6)	−1 (1.5)	0 (6.0)	0 (40)	0 (100)
12	1 (8)	1 (2.0)	−1 (5.0)	1 (50)	−1 (50)	41	−2 (2)	−1 (1.5)	0 (6.0)	0 (40)	0 (100)
13	−1 (4)	−1 (1.0)	1 (7.0)	1 (50)	−1 (50)	42	2 (10)	0 (1.5)	0 (6.0)	0 (40)	0 (100)
14	1 (8)	−1 (1.0)	1 (7.0)	1 (50)	−1 (50)	43	0 (6)	−2 (0.5)	0 (6.0)	0 (40)	0 (100)
15	−1 (4)	1 (2.0)	1 (7.0)	1 (50)	−1 (50)	44	0 (6)	2 (2.5)	0 (6.0)	0 (40)	0 (100)
16	1 (8)	1 (2.0)	1 (7.0)	1 (50)	−1 (50)	45	0 (6)	0 (1.5)	−2 (4.0)	0 (40)	0 (100)
17	−1 (4)	−1 (1.0)	−1 (5.0)	−1 (30)	1 (150)	46	0 (6)	0 (1.5)	2 (8.0)	0 (40)	0 (100)
18	1 (8)	−1 (1.0)	−1 (5.0)	−1 (30)	1 (150)	47	0 (6)	0 (1.5)	0 (6.0)	−2 (20)	0 (100)
19	−1 (4)	1 (2.0)	−1 (5.0)	−1 (30)	1 (150)	48	0 (6)	0 (1.5)	0 (6.0)	2 (60)	0 (100)
20	1 (8)	1 (2.0)	−1 (5.0)	−1 (30)	1 (150)	49	0 (6)	0 (1.5)	0 (6.0)	0 (40)	−2 (0)
21	−1 (4)	−1 (1.0)	1 (7.0)	−1 (30)	1 (150)	50	0 (6)	0 (1.5)	0 (6.0)	0 (40)	2 (200)
22	1 (8)	−1 (1.0)	1 (7.0)	−1 (30)	1 (150)	51–54	0 (6)	0 (1.5)	0 (6.0)	0 (40)	0 (100)

*, Combination number; •, real values (given in parentheses) are in % w/w; Factors X_1, X_2, X_3, X_4 and X_5 are solvent/bacterial bran ratio (S/BB), contact time, solvent pH, process temperature and shaking (RPM), respectively

enzyme source with 1.0% (w/v) pullulan at 75 °C in 2.0 ml of phosphate buffer (0.1 M, pH 6.0). Reducing sugars released were measured by 3,5 Dinitrosalycilic acid method [25]. A separate blank was setup for each sample to correct the non-enzymatic release of sugars. One unit of pullulanase was defined as the amount of the enzyme that released 1 μmol of reducing sugars as glucose min-1 under the standard assay conditions.

3
Results and discussion

C. thermosulfurogenes SV2 grew optimally at 60 °C and produced 910 U of thermostable pullulanase per litre culture broth in submerged fermentation [11, 12]. In our earlier studies, various nutrients were screened using Plackett–Burman design (a statistical design to screen '*N*–1' variables in only '*N*' experiments, where '*N*' is a multiple of four). By using this technique, the yield of the enzyme was improved from 1142 U per kg BB (when grown at 60 °C in 24 h on wheat bran that was moistened with distilled water) to 3948 U of thermostable pullulanase per kg BB, in one of the combinations [14]. In continuation to this study, we have employed response surface methodology, a central composite rotatable design, to optimize the levels of five selected nutrients for the enzyme production and the studies resulted in the production of 4322 U of thermostable pullulanase per kg BB [15].

3.1
Response surface analysis for the optimization of the process parameters

The actual yields obtained in the experiments and the model predicted yields of thermostable pullulanase are given in Table 2. The regression co-efficients and significance levels of the terms are given in Table 3. It is evident from Table 3 that the model used in the present study gave a satisfactory fit ($P < 0.0000$). The significant factors and their interactions were identified and considered for selecting the best fits. It can be seen from the degree of significance (Table 3) that the linear terms (Table 3) of solvent to bacterial bran ratio (S/BB) and shaking (RPM), and square terms of solvent pH were highly significant. As none of the process temperature and contact time terms was significant on the pullulanase leaching, their interactions with other process parameters are not discussed. From these observations, the equation for pullulanase leaching is given as:

$$\text{Pullulanase} = 4010.15 + 237.85x_1 + 233.65x_5 - 404.95x_3^2 \ ,$$

with a multiple correlation of 0.747, where x_1 = S/BB ratio [2.0–10.0 with 6.0 as central value], x_3 = solvent pH [4.0–8.0 with 6.0 as central value], x_5 = shaking (RPM) [0–200 with 100 as central value].

Table 2. Thermostable pullulanase yields both predicted and experimental, obtained with Central composite rotatable design (CCRD) for the leaching of thermostable pullulanase from *Clostridium thermosulfurogenes* SV2-fermented bran

C.No.*	Pullulanase leached (U/kg BB)			C.No.*	Pullulanase leached (U/kg BB)		
	Predicted yield	Actual yield	Residual		Predicted yield	Actual yield	Residual
1	2554	2234	−320	28	3706	3980	274
2	3228	2690	−538	29	3543	3722	179
3	2745	2578	−196	30	3616	3796	180
4	3623	3620	−3	31	3242	3520	278
5	2828	2350	−478	32	3519	3916	397
6	3442	3892	450	33	3844	4022	178
7	2936	3410	474	34	3844	3580	−264
8	3753	3956	203	35	3844	3910	66
9	3058	3634	576	36	3844	3818	−26
10	3351	3812	461	37	3844	3680	−164
11	3005	2978	−27	38	3844	3708	−136
12	3501	3258	−243	39	3844	4122	278
13	3082	3210	128	40	3844	3910	66
14	3316	2970	−346	41	2956	2844	−112
15	2946	2812	−134	42	3908	4020	112
16	3383	3226	−157	43	3817	3824	7
17	3352	3722	370	44	3911	3904	−7
18	3867	3978	111	45	2015	2216	201
19	3379	3428	49	46	2101	1900	−201
20	4097	4082	−15	47	3670	3988	318
21	3557	3720	163	48	3534	3216	−318
22	4013	3936	−77	49	3214	3280	66
23	3501	3210	−291	50	4148	4082	−66
24	4160	3604	−556	51	3678	3566	−112
25	3587	3080	−507	52	3678	3684	6
26	3720	3366	−354	53	3678	3720	42
27	3369	3312	−57	54	3678	3748	70

* Combination number

Table 3. Significance of regression coefficients of pullulanase leaching model

Variable	Reg. co-eff.	*t*-value	Significance level
Intercept	4010.15	21.12	
X_1 (S/BB)	237.85	4.36	***
X_2 (Contact time)	23.45	0.43	
X_3 (Solvent pH)	21.65	0.39	
X_4 (Process temperature)	−34.05	−0.62	
X_5 (Shaking, RPM)	233.65	4.28	***
X_1^2	−61.45	−1.03	
X_2^2	46.45	0.78	
X_3^2	−404.95	−6.79	***
X_4^2	−18.95	−0.31	
X_5^2	0.79	0.01	
X_1X_2	50.81	0.83	
X_1X_3	−14.93	−0.24	
X_1X_4	−95.31	−1.56	
X_1X_5	−39.81	−0.65	
X_2X_3	−20.68	−0.34	
X_2X_4	−61.03	−1.00	
X_2X_5	−41.06	−0.67	
X_3X_4	−62.31	−1.02	
X_3X_5	−17.06	−0.28	
X_4X_5	−67.43	−1.11	
Block	−165.92	−1.50	

Significant levels of regression coefficient are given as ***99.9%, **99.0% and *95% by *t*-test
'F-ratio' for the model was 13.94 (degrees of freedom were 20, 53) (F Prob. 0.0000), R^2 adj. 0.83684

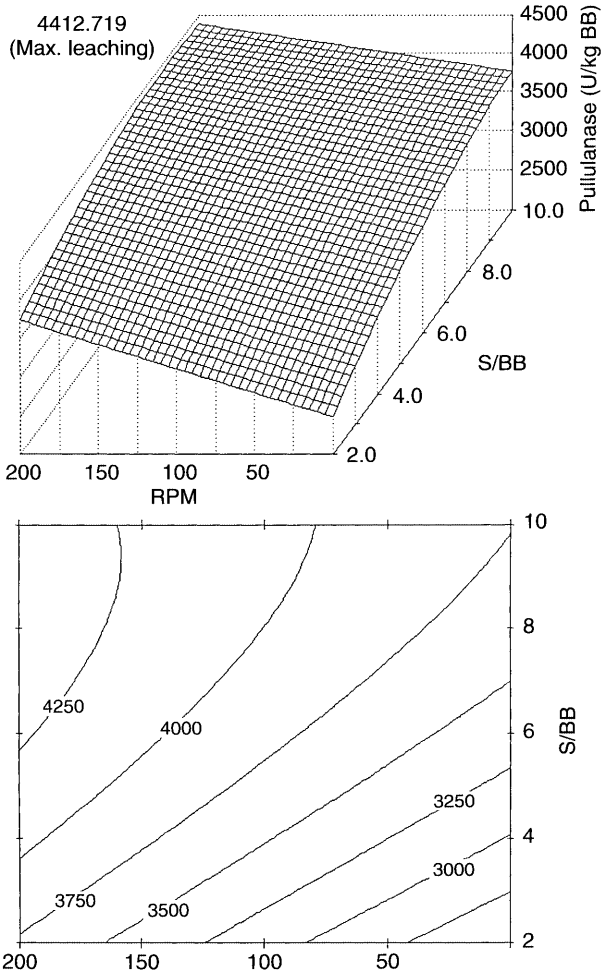

Fig. 1. Response surface plot (upper) and its contour plot of pullulanase leaching from *C. thermosulfurogenes* SV2-fermented bran: shaking (RPM) versus solvent to bacterial bran ratio (S/BB) with constant levels of contact time (0.5 h), process temperature (20 °C) and solvent pH 6.0

3.2 Interactions among the nutrients

Figures 1–3 are the response surface curves for variation in the amount of pullulanase leached, as a function of levels of two parameters with the other process parameter being at its optimum level as obtained through Analysis of Variance (ANOVA), and contact time and process temperature at their lowest levels. From the response surface plots and their contour plots, it is very easy and convenient to understand the interactions between the process parameters and also to locate their optimum levels. It can be seen from the response surface plots (Figs. 1, 2) that the leaching of pullulanase gradually increased upon increasing the shaking up to 200 RPM. A similar relationship was observed between S/BB ratio and the pullulanase leaching (Figs. 1, 3). The overall pullulanase leached out was very high at high levels of both shaking and S/BB (Fig. 1). The solvent pH 6.0 was found to be optimum for the leaching of pullulanase and any increase or decrease in the pH of the solvent resulted in sharp decrease in the leaching of pullulanase (Figs. 2, 3).

From the above observations it is clear that the higher levels of S/BB ratio and shaking, and the solvent pH 6.0 were favourable for the effective leaching of thermostable pullulanase from *C. thermosulfurogenes* SV2-fermented bran. When an optimization programme was run within the tested range by keeping the process temperature and contact time at their lowest levels, the optimum levels of the process parameters obtained were: S/BB ratio, 9.0; shaking, 200 RPM and solvent pH, 6.0. With these levels, the model has predicted 5029 U of thermostable pullulanase leaching per kg bacterial bran. When these values were experimentally verified, 4829 U of thermostable pullulanase was leached out per kg bacterial bran. To further validate and confirm these predictions, an experiment was designed with random but moderate levels of the process parameters (S/BB ratio, 4; shaking, 50 RPM and solvent pH, 6.0). Under these conditions, 2916 U of thermostable pullulanase was leached out per kg *C. thermosulfurogenes* SV2-fermented bran, which was only about 8% less than the model predicted value (the model has predicted 3162 U thermostable pullulanase per kg BB).

In the literature no reports are available on the systematic approach for process optimization for the effective leaching of the enzymes. The conventional process formulation studies are usually time consuming and expensive [14, 15, 19]. To overcome these problems, we have used response surface methodology for the optimization of process parameters for the effective leaching of thermostable pullulanase from *C. thermosulfurogenes* SV2-fermented bran. From the present study, it is evident that the

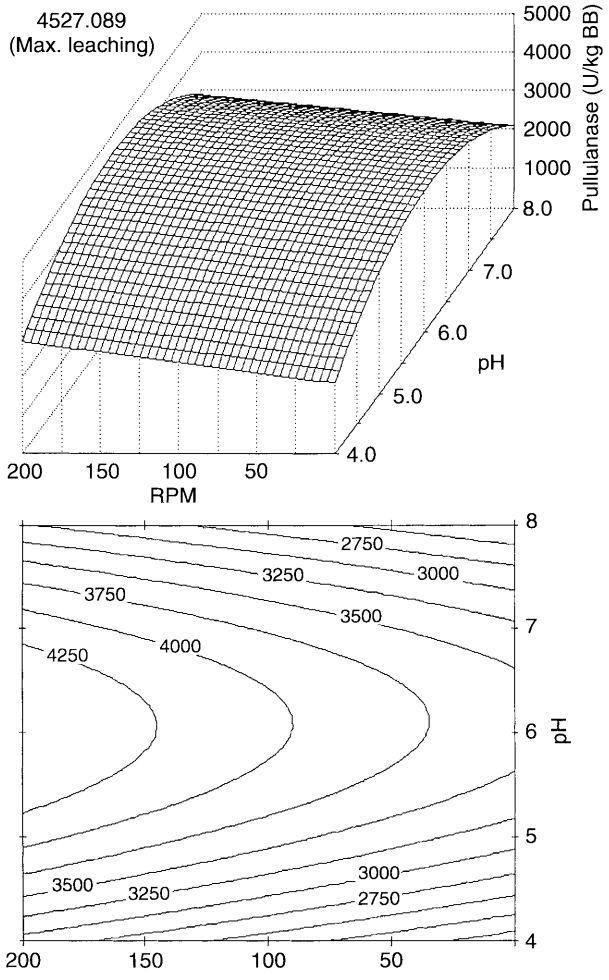

Fig. 2. Response surface plot (upper) and its contour plot of pullulanase leaching from *C. thermosulfurogenes* SV2-fermented bran: shaking (RPM) versus solvent pH with constant levels of contact time (0.5 h), process temperature (20 °C) and S/BB ratio (9.0)

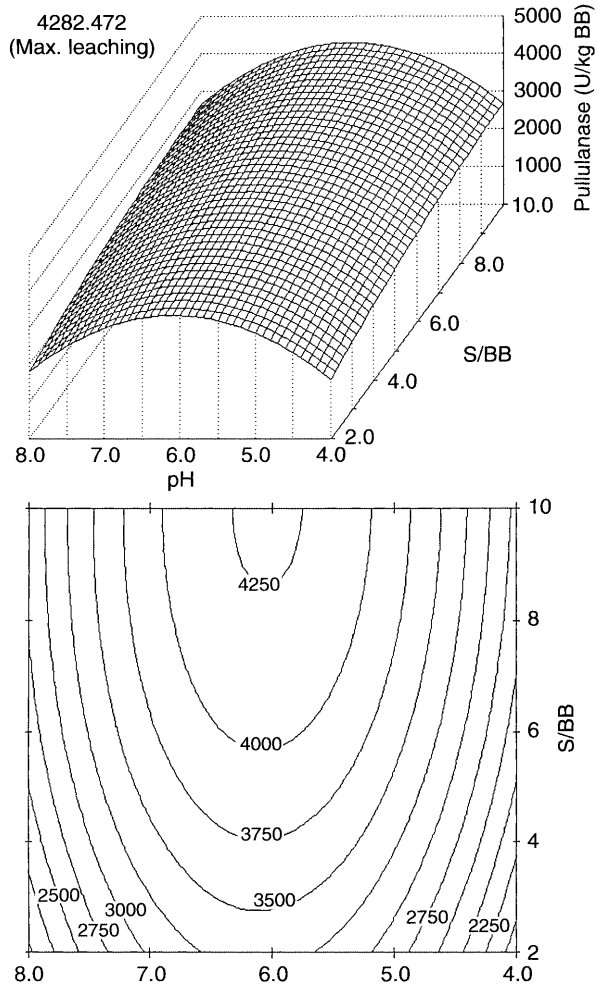

Fig. 3. Response surface plot (upper) and its contour plot of pullulanase leaching from *C. thermosulfurogenes* SV2-fermented bran: solvent pH versus solvent to bacterial bran ratio (S/BB) with constant levels of contact time (0.5 h), process temperature (20 °C) and shaking (200 PM)

use of response surface methodology not only helped us in identifying the significant process parameters and locating their optimum levels with minimum amount of resources and time, but also proved to be useful in increasing the pullulanase leaching by about 16%.

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