Bioprocess Engineering 21 (1999) 497–503 © Springer-Verlag 1999

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

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Abstract The optimization of nutrient levels for the production of thermostable pullulanase by *Clostridium thermosulfurogenes* SV2 in solid-state fermentation (SSF) 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 potato starch, magnesium chloride, ferrous sulfate, corn steep liquor and pearl millet flour as model factors. Among the five independent variables studied, except magnesium chloride, all the nutrients were found significant. 16.5% potato starch, 2.5% corn steep, 0.015% ferrous sulfate and 14% pearl millet flour have been found optimal for the production of thermostable pullulanase. The strain SV2 produced 10% more pullulanase in the nutritionally optimized solid-state fermentation medium containing only four nutrients.

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 amylolytic 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, 6, 7, 8, 9]. In this direction, we have isolated an 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, 14].

In recent times, the bacterial systems are increasingly investigated for the production of enzymes and metabolites by solid-state fermentation (SSF) [15]. 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 [16] and reported to be the most appropriate process for developing countries [17]. Earlier, the SSF has been employed for the production of thermostable α-amylase by *Bacillus licheniformis* [18, 19] and *B. megaterium* [20], α-amylase by *B. coagulans* [16], proteases [21], alpha-galactosidase [22], tannin acyl hydrolase [23] and pectinase [24] by *Aspergillus niger* and L-glutaminase by *Vibrio costicola* [25].

The optimal design of culture medium is a very important aspect in the development of fermentation processes. Experimental design techniques are very useful tools for this purpose, as they can provide statistical models which help in understanding the interactions among the nutrients at varying concentrations and in calculating the optimal concentration of each nutrient for a given target (i.e., maximal enzyme production) [26]. 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 [22, 27]. In spite of the above advantages, the statistical designs are applied to a limited number of fermentation processes [14, 21, 22, 26, 28]. In the present study, concentrations of significant nutrients among the five key nutrients that were selected [14] using Plackett-Burman design were optimized using the response surface methodology (RSM), a central composite rotatable design (CCRD), for the production of thermostable pullulanase by *C. thermosulfurogenes* SV2 in SSF.

Received: 4 January 1999

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The authors thank the Council of Scientific and Industrial Research (CSIR), New Delhi, India for the financial support. PRMR gratefully acknowledges the CSIR for providing Senior Research Fellowship (SRF).

Table 1. The central composite rotatable design for optimization of five nutrients (each at five levels) for the production of thermostable pullulanase by *C. thermosulfurogenes* SV2 in SSF

C.No[a]	Coded values and real values[b]				
	Factor x_1	Factor x_2	Factor x_3	Factor x_4	Factor x_5
1	−1 (10)	−1 (1.0)	−1 (0.06)	−1 (0.03)	−1 (10)
2	1 (20)	−1 (1.0)	−1 (0.06)	−1 (0.03)	−1 (10)
3	−1 (10)	1 (2.0)	−1 (0.06)	−1 (0.03)	−1 (10)
4	1 (20)	1 (2.0)	−1 (0.06)	−1 (0.03)	−1 (10)
5	−1 (10)	−1 (1.0)	1 (0.12)	−1 (0.03)	−1 (10)
6	1 (20)	−1 (1.0)	1 (0.12)	−1 (0.03)	−1 (10)
7	−1 (10)	1 (2.0)	1 (0.12)	−1 (0.03)	−1 (10)
8	1 (20)	1 (2.0)	1 (0.12)	−1 (0.03)	−1 (10)
9	−1 (10)	−1 (1.0)	−1 (0.06)	1 (0.06)	−1 (10)
10	1 (20)	−1 (1.0)	−1 (0.06)	1 (0.06)	−1 (10)
11	−1 (10)	1 (2.0)	−1 (0.06)	1 (0.06)	−1 (10)
12	1 (20)	1 (2.0)	−1 (0.06)	1 (0.06)	−1 (10)
13	−1 (10)	−1 (1.0)	1 (0.12)	1 (0.06)	−1 (10)
14	1 (20)	−1 (1.0)	1 (0.12)	1 (0.06)	−1 (10)
15	−1 (10)	1 (2.0)	1 (0.12)	1 (0.06)	−1 (10)
16	1 (20)	1 (2.0)	1 (0.12)	1 (0.06)	−1 (10)
17	−1 (10)	−1 (1.0)	−1 (0.06)	−1 (0.03)	1 (20)
18	1 (20)	−1 (1.0)	−1 (0.06)	−1 (0.03)	1 (20)
19	−1 (10)	1 (2.0)	−1 (0.06)	−1 (0.03)	1 (20)
20	1 (20)	1 (2.0)	−1 (0.06)	−1 (0.03)	1 (20)
21	−1 (10)	−1 (1.0)	1 (0.12)	−1 (0.03)	1 (20)
22	1 (20)	−1 (1.0)	1 (0.12)	−1 (0.03)	1 (20)
23	−1 (10)	1 (2.0)	1 (0.12)	−1 (0.03)	1 (20)
24	1 (20)	1 (2.0)	1 (0.12)	−1 (0.03)	1 (20)
25	−1 (10)	−1 (1.0)	−1 (0.06)	−1 (0.06)	1 (20)
26	1 (20)	−1 (1.0)	−1 (0.06)	1 (0.06)	1 (20)
27	−1 (10)	1 (2.0)	−1 (0.06)	1 (0.06)	1 (20)
28	1 (20)	1 (2.0)	−1 (0.06)	1 (0.06)	1 (20)
29	−1 (10)	−1 (1.0)	1 (0.12)	1 (0.06)	1 (20)
30	1 (20)	−1 (1.0)	1 (0.12)	1 (0.06)	1 (20)
31	−1 (10)	1 (2.0)	1 (0.12)	1 (0.06)	1 (20)
32	1 (20)	1 (2.0)	1 (0.12)	1 (0.06)	1 (20)
33–40	0 (15)	0 (1.5)	0 (0.09)	0 (0.045)	0 (15)
41	−2 (5)	0 (1.5)	0 (0.09)	0 (0.045)	0 (15)
42	2 (25)	0 (1.5)	0 (0.09)	0 (0.045)	0 (15)
43	0 (15)	−2 (0.5)	0 (0.09)	0 (0.045)	0 (15)
44	0 (15)	2 (2.5)	0 (0.09)	0 (0.045)	0 (15)
45	0 (15)	0 (1.5)	−2 (0.03)	0 (0.045)	0 (15)
46	0 (15)	0 (1.5)	2 (0.15)	0 (0.045)	0 (15)
47	0 (15)	0 (1.5)	0 (0.09)	−2 (0.015)	0 (15)
48	0 (15)	0 (1.5)	0 (0.09)	2 (0.075)	0 (15)
49	0 (15)	0 (1.5)	0 (0.09)	0 (0.045)	−2 (5)
50	0 (15)	0 (1.5)	0 (0.09)	0 (0.045)	2 (25)
51–54	0 (15)	0 (1.5)	0 (0.09)	0 (0.045)	0 (15)

[a] Combination number

[b] real values (given in parentheses) are in % w/w; Factors x_1, x_2, x_3, x_4 and x_5 are potato starch, corn steep liquor, magnesium chloride, ferrous sulfate and pearl millet flour, respectively

Table 2. Central composite rotatable design for the production of thermostable pullulanase by *C. thermosulfurogenes* SV2 in SSF: mathematically predicted yields and experimental yields

C. No[a]	Pullulanase (U/kg BB)		
	Predicted yield	Residual	Actual yields
1	3256	3070	186
2	2997	3190	−193
3	2559	2810	−251
4	3283	3250	33
5	3309	2950	359
6	2566	2980	−414
7	3003	2870	133
8	3244	3220	24
9	2633	2430	203
10	3088	3060	28
11	2004	2250	−246
12	2903	2930	−27
13	1597	1980	−383
14	4148	3810	338
15	3240	2920	320
16	2874	3120	−246
17	2722	2880	−158
18	3264	3230	34
19	3701	3760	−59
20	4067	3850	217
21	3463	3120	343
22	3322	3280	42
23	3213	3370	−157
24	3396	3520	−124
25	2470	2200	270
26	1766	2080	−314
27	3418	3110	308
28	2317	2620	−303
29	1743	1920	−177
30	3555	3210	345
31	3362	3150	212
32	3178	3270	−92
33	3234	3310	−76
34	2854	3120	−270
35	3854	3620	234
36	3174	3280	−106
37	2974	3180	−206
38	2374	2880	−506
39	3714	3550	164
40	4374	3880	494
41	1398	1820	−422
42	3875	3520	355
43	2364	2590	−226
44	3809	3650	159
45	3285	3120	165
46	2638	2870	−232
47	3202	3180	22
48	2041	2130	−89
49	3287	3190	97
50	2916	3080	−164
51	2362	2820	−458
52	3742	3510	232
53	3582	3430	152
54	4082	3680	402

[a] Combination number

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 [29].

Table 3. Significance of regression coefficients of pullulanase production model

Variable	Reg. Co-eff.	t-value	Significance level
Intercept	3493.25	19.43	
X_1 (Potato starch)	230.75	4.44	***
X_2 (Corn steep liquor)	168.75	3.25	***
X_3 (Magnesium chloride)	36.75	0.71	
X_4 (Ferrous sulfate)	−234.75	−4.50	***
X_5 (Pearl millet flour)	37.75	0.72	
X_1^2	−143.67	−2.5	*
X_2^2	−31.17	−0.55	
X_3^2	−62.42	−1.10	
X_4^2	−147.42	−2.60	*
X_5^2	−27.42	−0.48	
X_1X_2	−85.93	−1.48	
X_1X_3	75.93	1.31	
X_1X_4	75.56	1.32	
X_1X_5	− 85.31	−1.47	
X_2X_3	−7.81	−0.13	
X_2X_4	22.81	0.39	
X_2X_5	150.93	2.60	*
X_3X_4	107.18	1.84	
X_3X_5	7.81	0.13	
X_4X_5	−112.81	−1.94	
Block	−107.80	−1.02	

Significant levels of regression coefficients are given as ***99.9%, **99.0% and *95% by t-test
'F-ratio' for the model was 4.334 (degree of freedom were 20, 53) F Prob. 0.00011), R^2 adj. 0.56917

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 medium composed of 10 g of solid substrate and appropriate volume of moistening liquid containing the added nutrients. N2 was used as head space gas. Just before inoculations, a 2% (v/w) Na_2 S of 2.5% (w/v) solution was added to the medium to further maintain the reduced conditions. Care was taken to maintain the moisture level of the inoculated medium at 65%. 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 (28 ± 2 °C) with a contact time of 30 min and an agitation speed of 150 rpm on a rotary shaker. The extracts were clarified by squeezing through dampened cheese cloth [18] followed by centrifugation (8000 × g for 20 min) and the supernatant was used as enzyme source.

Five key nutrients, viz., magnesium chloride (0.03–0.15%), potato starch (5–25%), ferrous sulfate (15–75 ppm), pearl millet flour (5–25%) and corn steep liquor (0.5–2.5%), with 0.09%, 15%, 45 ppm, 15% and 1.5%, respectively, as their central points were studied. The concentration range for each nutrient was fixed based on the literature and on our own experience gained. Carbon and other nutrient sources were dissolved in appropriate amount of distilled water (moistening agent) and the pH was adjusted to 7.2 and then used for moistening the wheat bran before autoclaving. Corn steep liquor was prepared as 10X solution and added to the medium after separately autoclaved at 10 lbs for 10 min.

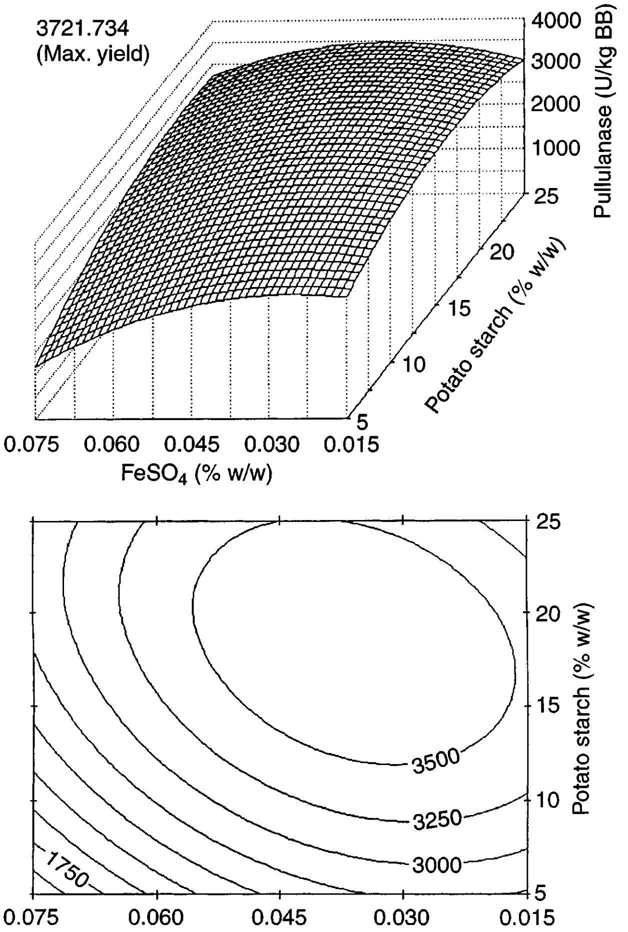

Fig. 1. Response surface plot (upper) and its contour plot of β-amylase production by *C. thermosulfurogenes* SV2: potato starch versus ferrous sulfate with constant levels of (% w/w): magnesium chloride (0.03), corn steep liquor (2.5) and pearl millet flour (14)

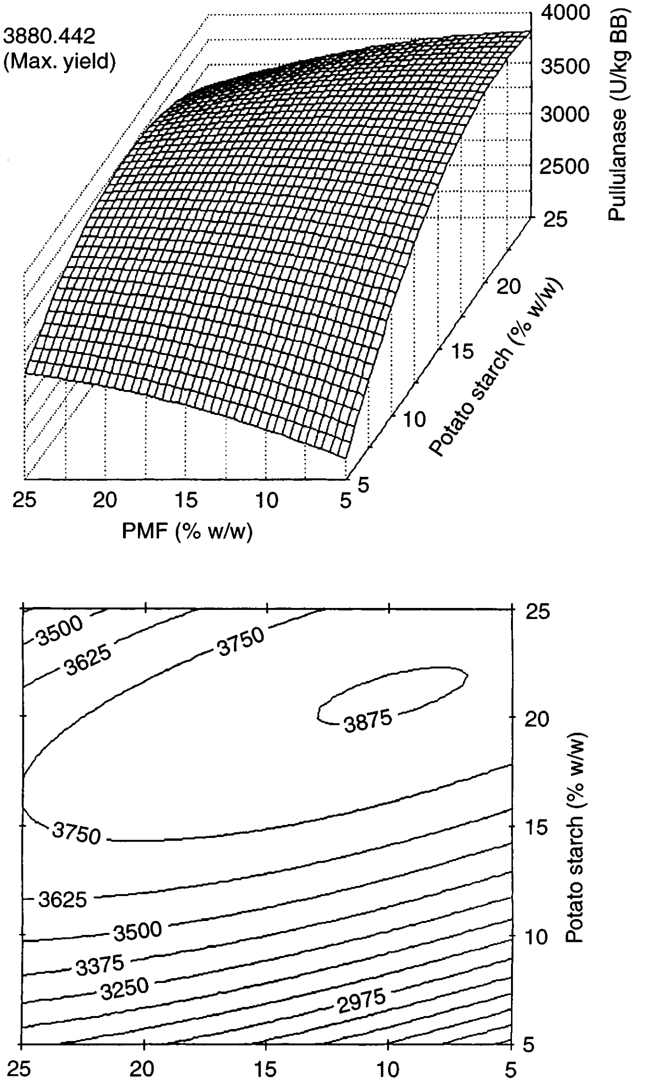

Fig. 2. Response surface plot (upper) and its contour plot of β-amylase production by *C. thermosulfurogenes* SV2: potato starch versus pearl millet flour with constant levels of (% w/w): magnesium chloride (0.03), corn steep liquor (2.5) and ferrous sulfate (0.015)

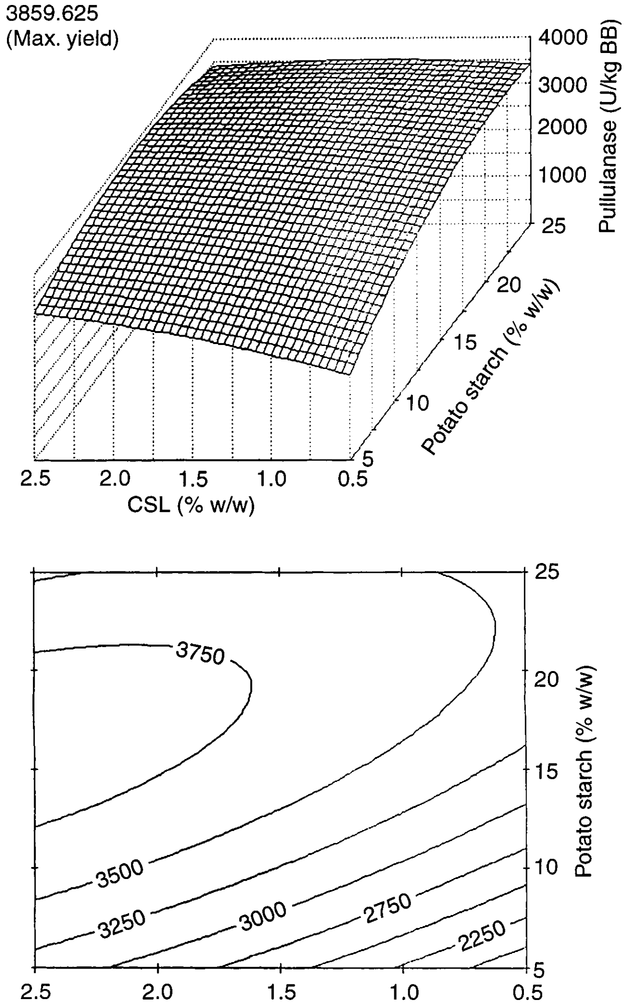

Fig. 3. Response surface plot (upper) and its contour plot of β-amylase production by *C. thermosulfurogenes* SV2: potato starch versus corn steep liquor with constant levels of (% w/w): magnesium chloride (0.03), ferrous sulfate (0.015) and pearl millet flour (14)

2.3
Experimental design

The experiments were conducted in a randomized fashion 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 to 40 and 51 to 54 involving the replications of the central points. Once the experiments are performed, the co-efficient of polynomial model is calculated using the equation [30]:

$$Y = b_0 + \Sigma b_i x_i + \leq \Sigma b_{ij} x_i x_j + \Sigma_i \leq \Sigma_j \leq \Sigma_k b_{ijk} x_i x_k + e \ ,$$

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 [27]. Model terms were selected or rejected based on the student *t*-value [31] or significance. Three-dimensional plots of two factors (at five different levels each) versus the enzyme yield 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 concentrations of two nutrients and their interactions on the yield of pullulanase by keeping the other three nutrients at their optimal concentrations (as obtained through ANOVA). From these three-dimensional plots, the interaction of one nutrient with other nutrients was studied. The optimum concentration of each nutrient was identified based on the hump in the three dimensional plot or from the central point of the corresponding contour plot.

2.5
Pullulanase assay

The pullulanase activity in the clarified samples was measured by incubating 0.5 ml appropriately diluted enzyme source with 1% (w/v) pullulan at 75 °C in 2 ml of

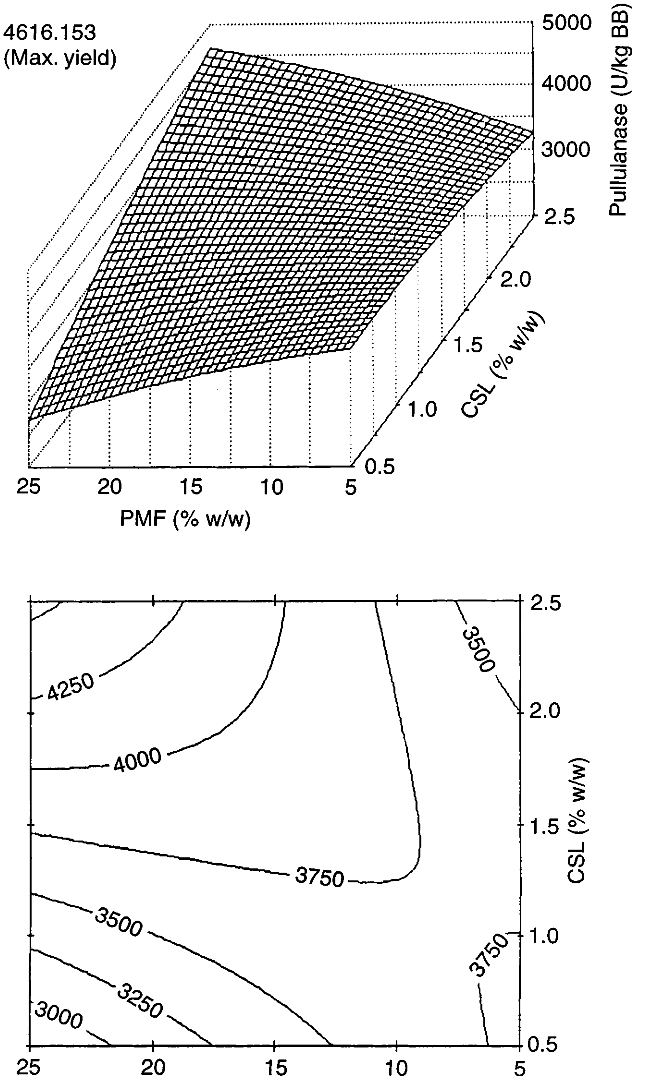

Fig. 4. Response surface plot (upper) and its contour plot of β-amylase production by *C. thermosulfurogenes* SV2: corn steep liquor versus pearl millet flour with constant levels of (% w/w): magnesium chloride (0.03), ferrous sulfate (0.015) and potato starch (16.5)

phosphate buffer (0.1 M, pH 6.0). Reducing sugars released were measured by a 3, 5 dinitrosalycilic acid method [32]. 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 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]. The strain SV2 produced on an average 1142 U of thermostable pullulanase per kilogram BB when grown at 60 °C in 24 h on wheat bran that was moistened with distilled water [13].

In our earlier studies, various nutrients were screened using Plackett-Burman design [13] (a statistical design to screen 'N-1' variables in only 'N' experiments, where N is a multiple of 4) and found magnesium chloride, potato starch, ferrous sulfate, pearl millet flour and corn steep liquor (CSL) as most effective in promoting the enzyme yields resulting in the production of 3948 U of thermostable pullulanase per kg BB, in one of the combinations [14].

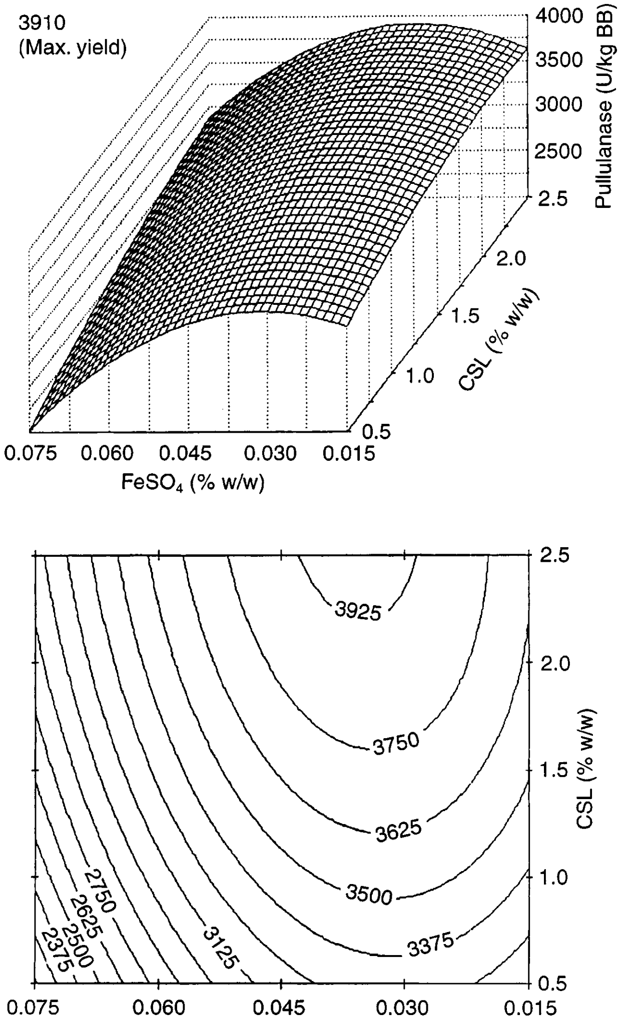

Fig. 5. Response surface plot (upper) and its contour plot of β-amylase production by *C. thermosulfurogenes* SV2: corn steep liquor versus ferrous sulfate with constant levels of (% w/w): magnesium chloride (0.03), potato starch (16.5) and pearl millet flour (14)

3.1
Response surface analysis for the optimization of nutrient levels

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 ($P < 0.00011$). 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 of potato starch, corn steep liquor and ferrous sulphate, square

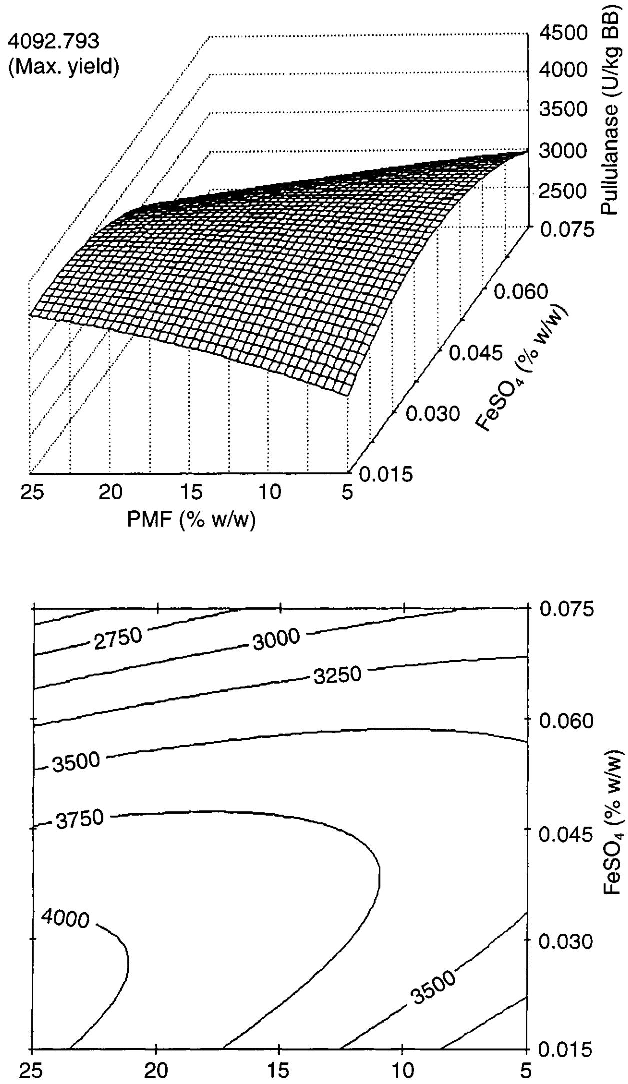

Fig. 6. Response surface plot (upper) and its contour plot of *β*-amylase production by *C. thermosulfurogenes* SV2: ferrous sulfate versus pearl millet flour with constant levels of (% w/w): magnesium chloride (0.03), corn steep liquor (2.5) and potato starch (14)

terms of potato starch and ferrous sulphate and interactive terms of pearl millet flour and corn steep liquor were highly significant. As none of the magnesium chloride terms was significant on the pullulanase production, its interactions with other nutrients are not discussed. From these observations, the equation for pullulanase is given by:

$$\text{Pullulanase} = 3493.25 + 230.75x_1 + 168.75x_2 - 234.75x_4 - 143.67x_1^2 - 147.42x_4^2 + 150.93x_2x_5 \ ,$$

with a multiple correlation of 0.739, where x_1 = potato starch [5%–25% with 15% central value], x_2 = corn steep liquor [0.5%–2.5% with 1.5% central value], x_4 = ferrous sulfate [0.15%–0.75% with 0.45% central value], x_5 = pearl millet flour [5%–25% with 15% central value].

3.2
Interactions among the nutrients

Figures 1–6 are the response surface curves for variation in the yields of pullulanase, as a function of concentrations of two nutrients with the other three nutrients being at their optimum levels (as obtained through Analysis of Variance). From the response surface plots and their contour plots, it is very easy and convenient to understand the interactions between two nutrients and also to locate their optimum levels. It can be seen from the response surface plots (Figs. 1, 2, 3) that the yield of pullulanase increased on increasing the concentration of potato starch up to about 20%, and the increase was more pronounced at lower concentrations of ferrous sulphate and pearl millet flour (Figs. 1, 2) and higher concentrations of corn steep liquor (Fig. 3).

Maximum yield of pullulanase was observed when the concentration of ferrous sulfate was about 0.03% and any increase in its concentration above this level resulted in decreased enzyme yield (Figs. 1, 5, 6). Therefore, ferrous sulfate should be incorporated at lower level for better production of the enzymes. A linear relationship was observed between the concentration of corn steep liquor and the yield of pullulanase (Figs. 3, 4, 5). Increased yields of pullulanase were observed at higher levels of corn steep liquor at both lower and higher levels of ferrous sulfate, however, with a sharp increase at lower levels of the latter (Fig. 5). This indicates that the higher levels of corn steep liquor and lower levels of ferrous sulfate are favorable for the highest production of pullulanase. For maximal production of pullulanase, if lower levels of ferrous sulphate (Fig. 6) and higher levels of corn steep liquor (Fig. 4) were selected, then higher levels of pearl millet flour concentration should be considered. In contrast, if higher concentration of potato starch was selected, then lower concentration of pearl millet flour should be considered (Fig. 2).

From the above observations it is clear that the maximum production of pullulanase was observed when the concentrations of potato starch, corn steep liquor, ferrous sulphate and pearl millet flour were at about 20, 2.5, 0.03 and 20%, respectively. When an optimization programme was run within the tested range, the optimum levels of the nutrients obtained were (% w/w): potato starch, 16.5; corn steep liquor, 2.5; ferrous sulphate, 0.025 and pearl millet flour, 14%, and with these levels, the model has predicted 4188 U of thermostable pullulanase per kg BB. When these values were experimentally verified, the strain SV2 produced 4322 U of thermostable pullulanase per kg BB. To further validate and confirm these predictions, an experiment was designed with random but moderate levels of the nutrients (potato starch, 10%; corn steep liquor, 1.5%; ferrous sulphate, 0.015% and pearl millet flour, 8%). The strain SV2 produced 2930 U of thermostable pullulanase per kg BB, which was only 8% less than the predicted yield (the model has predicted 3184 U of thermostable pullulanase per kg BB).

Earlier, there are few studies on the nutritional requirement of *C. thermosulfurogenes* for the production of thermostable pullulanase in SmF [6, 9, 33]. However, no reports are available in the literature concerning the systematic approach for the screening and optimization of nutritional requirement of the microorganisms for thermostable pullulanase production. The conventional medium formulation studies are usually time consuming and expensive [22]. To overcome these problems, we have used Plackett-Burman design to short-list few effective nutrients for the production of thermostable β-amylase by *C. thermosulfurogenes* SV2 in SSF and five nutrients were identified as most effective in promoting the enzyme yields [14]. From the present study, it is evident that the use of response surface methodology not only helped us in identifying the most significant nutrients and locating their optimum levels with minimum amount of resources and time, but also proved to be useful in increasing the yield of pullulanase by about 10% using only four nutrients.

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