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Optimization of thermostable amylopullulanase production in solid state fermentation by *Clostridium thermosulfurogenes* SVM17 through Plackett-Burman and response surface methodological approaches

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ABSTRACT

A total of fifteen nutrients comprising five carbon sources, four complex organic sources and three each of nitrogen and trace mineral sources were screened in a total of sixteen experiments using Plackett-Burman design for production of thermostable amylopullulanase by *Clostridium thermosulfurogenes* SVM17 in solid state fermentation (SSF) system. The design comprises screening of 'n−1' variables in 'n' number of experiments. Yield of enzyme was statistically analyzed to obtain for regression coefficients and *t*-values. Among fifteen nutrients screened, based on their effect in terms of product promoting ability, maltose, bajra flour, peptone, $CaCl_2 \cdot H_2O$ and $MnCl_2 \cdot 4H_2O$ were considered promising nutrients for enzyme production and selected for optimization of their concentration using response surface methodology based on the central composite rotatable design (CCRD). The design comprises a total of 54 experimental trials with first 32 organized in a fractional factorial design and experimental trials from 33–40 and 51–54 involving the replication of central points. The experimental trials from 41–50 are called star points or axial points. The design was applied to determine the effects of above medium components and their mutual interactions on amylopullulanase production. Within the tested range of concentrations, except $CaCl_2 \cdot 2H_2O$, all other components had significant effect on enzyme production. The optimum level of medium components for maximum production of the enzyme was (% w/w): maltose, 22.0; peptone, 0.8; $MnCl_2 \cdot 4H_2O$, 50 ppm; $CaCl_2 \cdot 2H_2O$, 15 ppm and bajra flour, 11.0.

Keywords: Clostridium thermosulfurogenes, amylopullulanase, solid state fermentation, Plackett-Burman design, response surface methodology

INTRODUCTION

Bioprocessing of starch into maltose and maltooligosaccharides by enzymatic means is gaining importance, because of their potential use in food, pharmaceutical, beverage and fine chemical industries (Fogarty and Kelly, 1990; Saha *et al*., 2009). So far reported amylases are thermally unstable and expensive (Hyun and Zeikus, 1985; Shen *et al.*, 1988). Therefore a high value is placed on extreme thermostable and thermoactive amylases in 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 more economical (Brown and Kelly, 1993). It is considered advantageous to have microorganisms that produce thermostable enzyme having properties of both amylase and pullulanase, because it cleaves both α-1,4 and α-1,6 linkages, respectively (Melasniemi, 1987; Spreinat and Antranikian, 1990; Dong *et al*., 1997; Ganghofner *et al*., 1998; Duffner, 2000; Gomes *et al*., 2003; Vishnu *et al*., 2006; Kim *et al*., 2008; Zareian *et al*., 2010). Therefore such type of endo acting enzymes have potential application for enhancement of starch saccharification process in industry (Ramesh *et al*., 1994). Thermoanaerobes show promise for the production of thermostable enzymes (Zeikus, 1979).

In this direction, anaerobic and thermophilic bacteria that secrete amylases were isolated in our laboratory (Swamy and Seenayya, 1996a). These strains were screened for production of amylolytic enzymes. The strain *Clostridium thermosulfurogenes* SVM17, which produced higher yield of the enzyme having properties of both amylase and pullulanase and stable at 100 °C was selected. Recently, the bacterial systems were investigated for production of enzymes and metabolites by solid state fermentation (SSF). These fermentation systems were considered to be closer to the natural habitats of microorganisms (Archana and Satyanarayana, 1997). The SSF has many advantages over submerged fermentation (SmF), including no need for complex machinery and sophisticated control systems; less volume of liquid required for product recovery, which could reduce the cost of downstream processing and subsequent waste treatment; usage of simple and cheap media for fermentation; lower energy demand, and often a high product yield; lower risk of contamination due to the inability of most contaminants to grow in absence of free flowing water (Ramesh and Lonsane, 1990; Pandey, 1992;

Gessesse and Mamo, 1999). Therefore the SSF process is considered to be more economical.

Selection of nutrients such as carbon, nitrogen and other nutrients is one of the most critical stages in an efficient and economic process development. The methodologies used for screening the nutrients fall into two categories; classical and statistical (Greasham, 1983). The application of statistical methodologies in fermentation process development has numerous advantages in terms of rapid and reliable short listing of nutrients, understanding the interactions among nutrients at varying concentrations and tremendous reduction in total number of experiments resulting in less time consumption, glassware, chemicals and man power (Srinivas *et al*., 1994; Rama Mohan Reddy *et al*., 1999a; Chauhan *et al.*, 2007; Reddy *et al*., 2008). Plackett-Burman design (Plackett and Burman, 1946) is a two level fractional factorial design and allows screening of up to 'n–1' variables in just 'n' number of experiments. In this design, generally a multiple of four i.e., 4, 8, 12, 16, 20, …., 4n experiments are required to screen 3, 7, 11, 15, 19, ….., 4n–1 components respectively, where 'n' is an integer. This design is employed for production of various metabolites and enzymes in submerged (Chauhan *et al*., 2007; Yu *et al*., 1997; Son *et al*., 1998; Khanna and Srivastava, 2005) and solid state fermentation (Srinivas *et al*., 1994; Rama Mohan Reddy *et al*., 1999a ; Ramana Murthy, 1994; Ramana Murthy *et al*., 1999; Cockshott and Sullivan Gary, 2001; Md Altaf *et al*., 2006).

Yield of any microbial product can be improved by optimization of medium components that are required in fermentation processes. Application of statistical methodologies in fermentation process development can result in improved yield of the product, reduced process variability, closer confirmation of the output response (product yield/ productivity) to normal and target requirements, reduced development time with overall costs. Conventional practice of single variable optimization is by maintaining other variables involved at a constant level. The major disadvantage of this 'change-single-factor-at-a-time' method is that it does not include interactive effects among the variables (Oh *et al*., 1995; Sen and Swaminathan, 1997; Satyanarayana *et al.*, 1999; Jaganatha Rao *et al*., 2000*)*. This method is a time consuming process and requires a number of experiments to determine optimum level, which are unreliable and therefore considered to be inferior to statistical methodologies. These limitations of a single factor optimization process can be eliminated by optimizing all the affecting parameters collectively by statistical experimental design using response surface methodology (RSM) (Box and Wilson*,* 1957; Khuri and Cornell, 1987; Jagannadha Rao, *et al*., 2000).

Today literature provides less information on the production of thermostable amylopullulanase in SSF. In the present study, we report the screening of nutrients using Plackett-Burman design and optimization of their concentration using response surface methodology

Table 1: Plackett-Burman design of experiment for screening of fifteen medium components for the production of thermostable amylopullulanase by *Clostridium thermosulfurogenes* SVM17 in solid state fermentation

Nutrients	Concentration* of nutrient (% w/w)		Combinations															
	'-' value	'+' value	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
Maltose	2.0	4.0	+	+	+	+	–	+	–	+	+	–	–	+	–	–	–	–
Xylose	2.0	4.0	–	+	+	+	+	–	+	–	+	+	–	–	+	–	–	–
Soluble starch	2.0	4.0	–	–	+	+	+	+	–	+	–	+	+	–	–	+	–	–
Sucrose	2.0	4.0	–	–	–	+	+	+	+	–	+	–	+	+	–	–	+	–
Lactose	2.0	4.0	+	–	–	–	+	+	+	+	–	+	–	+	+	–	–	–
Potato flour	2.0	4.0	–	+	–	–	–	+	+	+	+	–	+	–	+	+	–	–
Bajra flour	2.0	4.0	–	–	+	–	–	–	+	+	+	+	–	+	–	+	+	–
Jowar flour	2.0	4.0	+	–	–	+	–	–	–	+	+	+	+	–	+	–	+	–
Wheat flour	2.0	4.0	+	+	–	–	+	–	–	–	+	+	+	+	–	+	–	–
Corn steep liquor	0.5	1.0	–	+	+	–	–	+	–	–	–	+	+	+	+	–	+	–
Yeast extract	0.5	1.0	+	–	+	+	–	–	+	–	–	–	+	+	+	+	–	–
Peptone	0.5	1.0	–	+	–	+	+	–	–	+	–	–	–	+	+	+	+	–
$CaCl_2 . 2H_2O$	10 ppm	20 ppm	+	–	+	–	+	+	–	–	+	–	–	–	+	+	+	–
$MnCl_2 . 4H_2O$	10 ppm	20 ppm	+	+	–	+	–	+	+	–	–	+	–	–	–	+	+	–
NaCl	100 ppm	200 ppm	+	+	+	–	+	–	+	+	–	–	+	–	–	–	+	–

* '+' and '-' levels indicate the higher and lower levels, respectively, of a nutrient in that combination

Table 2: Yields of thermostable amylase and pullulanase by *C. thermosulfurogenes* SVM17 obtained in screening of fifteen nutrients using Plackett-Burman design in solid state fermentation

Combination number	Amylase activity (U/kg BB)		Pullulanase activity (U/kg BB)	
	Expt I	*Expt II*	*Expt I*	*Expt II*
1	17,071	16,547	24,698	26,241
2	17,930	16,983	26,410	27,332
3	17,452	17,669	26,389	25,919
4	17,693	16,701	25,893	27,049
5	15,937	15,220	23,897	24,161
6	18,398	17,757	27,080	28,158
7	18,968	18,909	28,442	28,409
8	17,829	17,542	26,417	26,060
9	17,071	16,972	25,922	26,967
10	16,692	16,043	24,588	25,298
11	14,036	14,152	20,486	21,387
12	15,933	15,532	26,293	26,684
13	14,605	14,830	22,425	21,157
14	14,984	15,649	21,779	22,801
15	13,277	13,118	19,856	19,686
16	14,226	14,091	21,344	21,502

Experiments were conducted in duplicates as per the design in 120 mL anaerobic serum vials containing 10 g of wheat bran with respective concentrations of nutrients (as shown in Table 1) dissolves in distilled water incubated at 60 °C for 72 h. The enzyme activities were assayed under standard conditions

Table 3: Regression coefficients and t-values obtained from the yields of amylase and pullulanase in the screening experiments in solid state fermentation

Nutrients	Amylase		Pullulanase	
	Reg. coeff	*t*-value	Reg. coeff	t-value
INTERCEPT	16242.00	287.19	24710.00	263.43
Maltose	946.00	16.73	1760.00	18.75
Xylose	608.00	10.76	930.00	9.92
Soluble starch	238.00	4.22	130.00	1.33
Sucrose	-16.00	-0.29	310.00	3.34
Lactose	495.00	8.77	915.00	9.76
Potato flour	421.00	7.44	366.00	3.91
Bajra flour	235.00	4.16	383.00	4.09
Jowar flour	-359.00	-6.36	-702.00	-7.49
Wheat flour	-195.00	-3.46	-26.00	-0.28
Corn steep liquor	-342.00	-6.05	-388.00	-4.14
Yeast extract	50.00	0.88	43.00	0.46
Peptone	-386.00	-6.82	-466.00	-4.97
$CaCl_2 \cdot 2H_2O$	-208.00	-3.67	-514.00	-5.48
$MnCl_2 \cdot 4H_2O$	423.00	7.49	522.00	5.57
NaCl	173.00	3.05	26.00	0.28

Regression coefficients and t-values were obtained by subjecting the enzyme yields (Table 2) to statistical analysis using 'Indostat' statistical software

for production of thermostable amylopullulanase by *Clostridium thermosulfurogenes* SVM17 in SSF.

MATERIALS AND METHODS

Microorganism

The bacterial strain used in the present study was isolated in our laboratory (Swamy and Seenayya, 1996a) and identified as *Clostridium thermosulfurogenes* SVM17.

Solid state fermentation technique

Solid state fermentation was carried out in 120 mL serum vials that contained 10 g of wheat bran moistened with appropriate volume of distilled water containing the required concentration of nutrients. The medium was flushed with nitrogen gas to create anaerobic conditions and vials were sealed and sterilized at 121 °C for 15 min. After cooling to room temperature (28 ± 2 °C), a 2% (v/w) of 2.5% (w/v) Na_2S solution was added to maintain further

Table 4: Central composite rotatable design (CCRD) of five medium components in coded and uncoded units: Effect of each combination on the production of thermostable amylopullulanase by *C. thermosulfurogenes* SVM17 in SSF

Combination number	Maltose % (w/w) X_1	Peptone % (w/w) X_2	$MnCl_2 \cdot 4H_2O$ ppm X_3	$CaCl_2 \cdot 2H_2O$ ppm X_4	Bajra flour % (w/w) X_5
1	-1 (7.0)	-1 (0.6)	-1 (30.0)	-1 (10.0)	-1 (7.0)
2	1 (17.0)	-1 (0.6)	-1 (30.0)	-1 (10.0)	-1 (7.0)
3	-1 (7.0)	1 (1.0)	-1 (30.0)	-1 (10.0)	-1 (7.0)
4	1 (17.0)	1 (1.0)	-1 (30.0)	-1 (10.0)	-1 (7.0)
5	-1 (7.0)	-1 (0.6)	1 (70.0)	-1 (10.0)	-1 (7.0)
6	1 (17.0)	-1 (0.6)	1 (70.0)	-1 (10.0)	-1 (7.0)
7	-1 (7.0)	1 (1.0)	1 (70.0)	-1 (10.0)	-1 (7.0)
8	1 (17.0)	1 (1.0)	1 (70.0)	-1 (10.0)	-1 (7.0)
9	-1 (7.0)	-1 (0.6)	-1 (30.0)	1 (20.0)	-1 (7.0)
10	1 (17.0)	-1 (0.6)	-1 (30.0)	1 (20.0)	-1 (7.0)
11	-1 (7.0)	1 (1.0)	-1 (30.0)	1 (20.0)	-1 (7.0)
12	1 (17.0)	1 (1.0)	-1 (30.0)	1 (20.0)	-1 (7.0)
13	-1 (7.0)	-1 (0.6)	1 (70.0)	1 (20.0)	-1 (7.0)
14	1 (17.0)	-1 (0.6)	1 (70.0)	1 (20.0)	-1 (7.0)
15	-1 (7.0)	1 (1.0)	1 (70.0)	1 (20.0)	-1 (7.0)
16	1 (17.0)	1 (1.0)	1 (70.0)	1 (20.0)	-1 (7.0)
17	-1 (7.0)	-1 (0.6)	-1 (30.0)	-1 (10.0)	1 (17.0)
18	1 (17.0)	-1 (0.6)	-1 (30.0)	-1 (10.0)	1 (17.0)
19	-1 (7.0)	1 (1.0)	-1 (30.0)	-1 (10.0)	1 (17.0)
20	1 (17.0)	1 (1.0)	-1 (30.0)	-1 (10.0)	1 (17.0)
21	-1 (7.0)	-1 (0.6)	1 (70.0)	-1 (10.0)	1 (17.0)
22	1 (17.0)	-1 (0.6)	1 (70.0)	-1 (10.0)	1 (17.0)
23	-1 (7.0)	1 (1.0)	1 (70.0)	-1 (10.0)	1 (17.0)
24	1 (17.0)	1 (1.0)	1 (70.0)	-1 (10.0)	1 (17.0)
25	-1 (7.0)	-1 (0.6)	-1 (30.0)	1 (20.0)	1 (17.0)
26	1 (17.0)	-1 (0.6)	-1 (30.0)	1 (20.0)	1 (17.0)
27	-1 (7.0)	1 (1.0)	-1 (30.0)	1 (20.0)	1 (17.0)
28	1 (17.0)	1 (1.0)	-1 (30.0)	1 (20.0)	1 (17.0)
29	-1 (7.0)	-1 (0.6)	1 (70.0)	1 (20.0)	1 (17.0)
30	1 (17.0)	-1 (0.6)	1 (70.0)	1 (20.0)	1 (17.0)
31	-1 (7.0)	1 (1.0)	1 (70.0)	1 (20.0)	1 (17.0)
32	1 (17.0)	1 (1.0)	1 (70.0)	1 (20.0)	1 (17.0)
33	0 (12.0)	0 (0.8)	0 (50.0)	0 (15.0)	0 (12.0)
34	0 (12.0)	0 (0.8)	0 (50.0)	0 (15.0)	0 (12.0)
35	0 (12.0)	0 (0.8)	0 (50.0)	0 (15.0)	0 (12.0)
36	0 (12.0)	0 (0.8)	0 (50.0)	0 (15.0)	0 (12.0)
37	0 (12.0)	0 (0.8)	0 (50.0)	0 (15.0)	0 (12.0)
38	0 (12.0)	0 (0.8)	0 (50.0)	0 (15.0)	0 (12.0)
39	0 (12.0)	0 (0.8)	0 (50.0)	0 (15.0)	0 (12.0)
40	0 (12.0)	0 (0.8)	0 (50.0)	0 (15.0)	0 (12.0)
41	-2 (2.0)	0 (0.8)	0 (50.0)	0 (15.0)	0 (12.0)
42	2 (22.0)	0 (0.8)	0 (50.0)	0 (15.0)	0 (12.0)
43	0 (12.0)	-2 (0.4)	0 (50.0)	0 (15.0)	0 (12.0)
44	0 (12.0)	2 (1.2)	0 (50.0)	0 (15.0)	0 (12.0)
45	0 (12.0)	0 (0.8)	-2 (10.0)	0 (15.0)	0 (12.0)
46	0 (12.0)	0 (0.8)	2 (90.0)	0 (15.0)	0 (12.0)
47	0 (12.0)	0 (0.8)	0 (50.0)	-2 (5.0)	0 (12.0)
48	0 (12.0)	0 (0.8)	0 (50.0)	2 (25.0)	0 (12.0)
49	0 (12.0)	0 (0.8)	0 (50.0)	0 (15.0)	-2 (2.0)
50	0 (12.0)	0 (0.8)	0 (50.0)	0 (15.0)	2 (22.0)

(continued)

51	0 (12.0)	0 (0.8)	0 (50.0)	0 (15.0)	0 (12.0)
52	0 (12.0)	0 (0.8)	0 (50.0)	0 (15.0)	0 (12.0)
53	0 (12.0)	0 (0.8)	0 (50.0)	0 (15.0)	0 (12.0)
54	0 (12.0)	0 (0.8)	0 (50.0)	0 (15.0)	0 (12.0)

Uncoded units are given in parentheses

reduced conditions followed by 2 mL of 24 h old inoculum. Contents in the vials were mixed thoroughly and incubated at 60 °C in horizontal position. During incubation, the contents in vials were periodically mixed by gentle shaking and accumulated gases were removed using a sterile needle. At the end of incubation time (72 h), vials were taken out and enzyme was extracted from each vial with 0.1 M sodium acetate buffer (pH 5.5) at 1:5 w/v ratio 28 ± 2 °C with a contact time of 1 h at an agitation speed of 150 rpm on a rotary shaker. Extracts were clarified by squeezing through dampened cheese cloth (Ramesh and Lonsane, 1990), followed by centrifugation at 8000 rpm for 20 min at 4 °C, and the supernatant was used as source of extra cellular enzyme.

Screening of nutrients using Plackett-Burman design

A total of fifteen nutrients comprising of five carbon source (maltose, xylose, soluble starch, sucrose and lactose), four complex organic source (potato flour, bajra flour, jowar flour and wheat flour) and three each of nitrogen (corn steep liquor, yeast extract and peptone) and trace mineral sources ($CaCl_2 \cdot 2H_2O$, $MnCl_2 \cdot 4H_2O$ and NaCl) were screened in a total of sixteen experiments using Plackett-Burman design (Table 1).

Concentration for each nutrient was fixed based on the literature and on our own experience gained (Rama Mohan Reddy *et al*., 1999). All nutrients except monosaccharide, disaccharides and corn steep liquor were dissolved in appropriate amount of distilled water (moistening agent), pH was adjusted to 7.5 and then used for moistening the wheat bran before sterilization. Monosaccharide, disaccharide sugars and corn steep liquor were prepared at a concentration of 10X solutions and sterilized separately by autoclaving at 10 psi for 10 min and required concentration was added to the medium before inoculation. Care was taken to maintain the moisture level of inoculated medium at 73%. The data on the yields of amylase and pullulanase in these sixteen experiments were subjected to compatible analysis (Plackett and Burman, 1946) to obtain regression coefficients and *t*-values. The 'Indostat' statistical package was used for the data analysis. The nutrients with highest *t-value*s were considered as the best nutrients and thus selected for further optimization studies (Rama Mohan Reddy *et al.*, 1999b)

Optimization of concentration of nutrients using RSM

A central composite rotatable design (CCRD) was used to optimize the concentrations of nutrients. The design contains a total of 54 experimental trials with first 32 organized in a fractional factorial design (Cochran and Cox, 1957), the experimental trials from 33–40 and 51–54 involve the replications of central points and experimental trials from 41–50 are axial points (star points). The response i.e., amount of enzyme produced by *C. thermosulfurogenes* SVM17 was assumed to be influenced by the five factors selected for the study. Once the experiments were performed, the coefficients of second-order polynomial model for five factors were calculated from the following equation (Montgomery, 1991).

$$Y= b_0 + b_1X_1 + b_2X_2 + b_3X_3 + b_4X_4 + b_5X_5 + b_{11}X_1^2 + b_{22}X_2^2 + b_{33}X_3^2 + b_{44}X_4^2 + b_{55}X_5^2 + b_{12}X_1X_2 + b_{13}X_1X_3 + b_{14}X_1X_4 + b_{15}X_1X_5 + b_{23}X_2X_3 + b_{24}X_2X_4 + b_{25}X_2X_5 + b_{34}X_3X_4 + b_{35}X_3X_5 + b_{45}X_4X_5 + B$$

Where, y is the response (predicted yield of enzyme), b_0 is the intercept, b_1, b_2, b_3, b_4 and b_5 are the linear coefficients, b_{11}, b_{22}, b_{33}, b_{44} and b_{55} are the quadratic coefficients and $b_{12–15}$, $b_{23–25}$, $b_{34–35}$ and b_{45} are the interactive coefficients.

Significance of the model is determined based on lack of fit and significance of each coefficient was determined using the student *t*-test (Gong and Chen, 1998; Rama Mohan Reddy *et al.*, 2000). Graphical representation of these equations are called response surface curves, used to describe the individual and cumulative effect of the test variable (factor) on the response and to determine the mutual interactions between two test variables and their subsequent effect on the response (Khuri and Cornell, 1987; Montgomery, 1991). The three dimensional response surface plot was drawn with vertical axis representing the enzyme yield and two horizontal axes representing five different levels of two explanatory nutrients by keeping other three factors at zero level. The results were analyzed using the 'Indostat' statistical package. Optimum concentration of each nutrient is identified based on the hump in three dimensional plot.

Enzyme assays

The extracellular amylase and pullulanase activities from the clarified samples were measured by incubating 0.5 mL appropriately diluted enzyme sample with 0.5 mL of 1% (w/v) starch solution and pullulan solution in 2 mL of 0.1 M acetate buffer (pH 5.5) at 70 °C for 30 min, respectively. After incubation, the reaction was stopped by cooling the tubes in an ice bath. The reducing sugars released by enzymatic hydrolysis of soluble starch and pullulan were determined by addition of 1 mL of 3, 5-dinitrosalicylic acid (Miller, 1959). A separate blank was set up for each sample to correct the non enzymatic release of sugars. One unit of amylase or pullulanase is defined as the rate

Table 5: Experimental and predicted pullulanase and amylase activities of thermostable amylopullulanase produced by *C. thermosulfurogenes* SVM17 in solid state fermentation

Combination number	Pullulanase activity (U/kg BB)			Amylase activity (U/kg BB)		
	Experimental yield	Predicted yield	residual	Experimental yield	Predicted Yield	Residual
1	19,840	19,512	328	10,635	13,169	-2534
2	25,260	24,093	1167	21,432	19,916	1516
3	21,800	22,089	-289	15,400	15,340	60
4	26,400	25,202	1198	19,700	19,714	-14
5	19,400	18,689	711	14,440	13,425	1015
6	23,650	23,919	-269	18,600	20,213	-1613
7	22,550	20,950	1600	16,730	15,046	1684
8	22,780	24,711	-1931	18,760	19,461	-701
9	19,700	18,783	917	12,740	12,862	-122
10	27,050	25,403	1647	18,950	19,109	-159
11	20,120	19,934	186	13,760	14,531	-771
12	24,950	25,085	-135	19,020	18,404	616
13	19,320	19,361	-41	12,790	13,370	-580
14	26,240	26,630	-390	19,740	19,658	82
15	21,650	20,196	1454	13,240	14,489	-1249
16	26,200	25,996	204	18,190	18,403	-213
17	20,830	20,629	201	14,330	14,337	-7
18	24,010	24,144	-134	20,390	19,934	456
19	22,570	22,310	260	16,310	15,151	1159
20	23,350	24,356	-1006	18,080	18,374	-294
21	18,860	19,812	-952	13,980	14,600	-620
22	23,700	23,976	276	21,380	20,238	1142
23	20,890	21,177	-287	13,360	14,864	-1504
24	23,320	23,872	552	18,900	18,128	772
25	20,850	18,451	358	14,350	13,626	724
26	20,760	24,005	2399	17,380	18,721	-1341
27	18,780	18,706	-3245	13,860	13,937	-77
28	24,000	22,791	74	16,320	16,659	-339
29	18,600	19,035	1209	13,710	14,141	-431
30	26,490	25,238	-435	18,650	19,278	- 628
31	17,320	18,974	1252	13,850	13,901	-51
32	22,690	23,708	1654	18,100	16,665	1435
33	25,200	26,073	-1018	17,610	18,403	-793
34	23,590	26,073	-873	18,100	18,403	-303
35	27,110	16,282	-1323	17,720	18,403	-683
36	21,800	25,570	-1962	19,450	18,403	1047
37	24,750	21,606	1413	18,810	18,403	407
38	21,000	22,653	-1166	18,920	18,403	517
39	22,380	22,353	617	19,780	18,403	1377
40	23,400	24,310	- 910	19,420	18,403	1017
41	14,320	16,663	-2343	13,620	12,291	1329
42	27,010	25,987	1032	21,120	21,802	-682
43	20,440	21,987	-1547	20,860	19,633	1227
44	23,270	23,034	236	18,612	19,192	-580
45	20,240	22,734	-2494	17,620	17,380	240
46	24,010	22,827	1183	18,050	17,642	408
47	25,060	25,052	8	19,460	20,041	-581
48	22,840	24,159	-1319	19,500	18,271	1229
49	20,140	23,426	-3286	17,410	16,241	1169
50	24,230	22,255	1975	15,150	15,671	-521
51	26,180	24,540	1637	19,240	19,297	-57

(continued)

52	26,940	24,540	2397	19,450	19,297	153
53	25,930	24,543	1387	17,590	19,297	-1707
54	25,680	24,543	1137	17,670	19,297	-1627

Experiments were conducted in 120 mL anaerobic serum vials containing 10 g of wheat bran with respective concentrations of nutrients (Table 1) dissolved in distilled water, incubated at 60 °C for 72 h. The enzyme activities were assayed under standard assay conditions

of formation (1 µmole) of reducing sugars (as glucose equivalents) per min under standard assay conditions.

RESULTS AND DISCUSSION

C. thermosulfurogenes SVM17 grew optimally at 60 °C, and produced 2,600 and 1,300 U of thermostable amylase and pullulanase activities, respectively per liter of culture broth in submerged fermentation. In solid state fermentation, the strain SVM17 produced on an average of 9,221 and 10,080 U of thermostable amylase and pullulanase, respectively per kilogram of moistened bacterial bran (BB) grown at 60 °C in 72 h.

Effect of nutrients on enzyme production

The enzyme yields of *C. thermosulfurogenes* SVM17 from screening of fifteen nutrients in sixteen experiments using Plackett-Burman design are shown in Table 2. It is clear from the Table 2 that the strain produced highest average yields of amylase (18,968 U/kg BB) and pullulanase (28,426 U/kg BB) in combinations 7 of the design, followed by 6 and 2. The data on enzyme yields were subjected to statistical analysis to obtain regression coefficients and *t-value*s (Table 3). From Table 3, it is clear that maltose, xylose, lactose, potato flour, bajra flour, jowar flour, peptone, CSL, calcium chloride and manganous chloride showed significant effects on yields of both amylase and pullulanase. Apart from these, sucrose, soluble starch, wheat flour and sodium chloride were also significant on amylase synthesis but not on pullulanase synthesis. Effect of yeast extract was significant on synthesis of both the enzymes. Among carbon sources screened, maltose and xylose were found to be the best for maximum enzyme production, followed by lactose. Soluble starch influenced synthesis of amylase but not pullulanase production. Sucrose influenced pullulanase but not on amylase production. Since maltose greatly influenced both the enzymes production, it was selected for optimization studies. Among complex organic sources, potato flour and bajra flour showed positive effects on enzyme production. Therefore, bajra flour was selected for further optimization studies. Among nitrogen sources screened, peptone showed greater effect on enzyme production. Calcium chloride and manganous chloride were highly significant among trace mineral sources screened and therefore included for optimization.

Literature gives no information on the production of thermostable amylopullulanase in SSF. Maltose was reported to be the inducer of β-amylase production by *C. thermocellum* SS8 (Swamy *et al*., 1994) and *C. thermosulfurogenes* (Hyun and Zeikus, 1985a). In contrast, maltose was reported to be repressor for production of amylase by *Bacillus stearothermophilus* (Srivastava and Baruah, 1986). Swamy and Seenayya (1996b) reported that corn steep liquor in combination with yeast extract were the best nitrogen source for thermostable α-amylase and pullulanase production in SmF by *C. thermosulfurogenes* SV9. The significant effect of corn steep liquor on enzyme production could be due to presence of growth factors in it (Srinivas *et al*., 1994).

To identify the most important factor, screening of medium components adopting Plackett-Burman design have been employed for production of fungal proteinase (Ramana Murthy, 1994), cyclosporin A (Ramana Murthy *et al*., 1999), β-amylase and pullulanase (Rama Mohan Reddy *et al*., 1999b), echinocandin B (Cockshott and Sullivan Gary, 2001), biohydrogen (Pan *et al.*, 2008) and alkaline protease (Reddy *et al*., 2008).

Response surface analysis for the optimization of levels of medium components

The uncoded (natural) and coded (real) values of five medium components are given in Table 4, which gives various concentrations to be attempted. Mathematical relationship of the response Y_1 (pullulanase activity) and Y_2 (amylase activity) on these variables were calculated by second order polynomial equations.

$$Y_1 \text{ (Pullulanase activity)} = 26650.8 + 328.80X_1 + 261.70X_2 + 23.20X_3 - 223.20X_4 - 292.70X_5 - 1139.10X_1^2 - 841.60X_2^2 - 774.11X_3^2 - 317.80X_4^2 - 759.10X_5^2 - 367.20X_1X_2 + 162.205X_1X_3 + 509.70X_1X_4 - 266.60X_1X_5 - 79.10X_2X_3 - 356.60X_2X_4 - 224.10X_2X_5 + 350.3X_3X_4 + 16.0X_3X_5 - 362.20X_4X_5 - 577.40$$

$$Y_2 \text{ (amylase activity)} = 17508.20 + 2377.7X_1 - 110.3X_2 + 65.6X_3 - 442.4X_4 - 142.4X_5 - 562.6X_1^2 + 28.90X_2^2 - 446.40X_3^2 - 35.10X_4^2 - 835.10X_5^2 - 593.3X_1X_2 + 10.40X_1X_3 - 125.2X_1X_4 - 287.7X_1X_5 - 137.60X_2X_3 - 125.70X_2X_4 - 339.50X_2X_5 + 63.00X_3X_4 + 1.80X_3X_5 - 101.3X_4X_5 - 894.30$$

where, Y_1 and Y_2 are the predicted pullulanase and amylase activities, respectively. X_1, X_2, X_3, X_4 and X_5 are the coded values of maltose, peptone, manganous chloride, calcium chloride and bajra flour, respectively.

Table 6: Significance of regression coefficients of pullulanase activity (in SSF) model*

Process variable	Regression coefficient	Computed t-value	Probability	Significance Level
INTERCEPT	26650.80	19.26	0.0000	
X_1 Maltose	328.80	8.59	0.0000	* * *
X_2 Peptone	261.70	0.97	0.3430	
X_3 $MnCl_2$ $4H_2O$	23.20	0.09	0.9323	
X_4 $CaCl_2$ $2H_2O$	-223.20	-0.82	0.4175	
X_5 Bajra flour	-292.70	-1.08	0.2899	
X_1^2	-1139.10	-3.51	0.0016	* * *
X_2^2	-841.60	-2.60	0.0153	*
X_3^2	-774.11	-2.39	0.0245	*
X_4^2	-317.80	-0.98	0.3359	
X_5^2	-759.10	-2.34	0.0271	*
X_1X_2	-367.20	-1.21	0.2364	
X_1X_3	162.20	0.54	0.5970	
X_1X_4	509.70	1.68	0.1045	
X_1X_5	-266.60	-0.87	0.3870	
X_2X_3	-79.10	-0.26	0.7962	
X_2X_4	-356.60	-1.18	0.2499	
X_2X_5	-224.10	-0.74	0.4662	
X_3X_4	350.30	1.16	0.2581	
X_3X_5	16.00	0.01	0.9959	
X_4X_5	-362.20	-1.19	0.2427	
Block	-577.40	- 0.91	0.3722	

*Significance levels of regression coefficients are given as *** 99.9% , ** 99.0% and * 95.0% by *t*-test
Regression coefficients, *t*-values and P values of pullulanase activity were obtained by subjecting the pullulanase yields (Table 5) to statistical analysis using 'Indostat' statistical software.

The predicted and experimental yield of pullulanase and amylase for 54 experiments are given in Table 5. The regression coefficients and *t-value*s of pullulanase and amylase are given in Tables 6 and 7, respectively. Analysis of variance (ANOVA) is required to test the significance and adequacy of the model. Summary of analysis of variance for evaluation of second order polynomial model for pullulanase and amylase are presented in Tables 8 and 9, respectively. From the ANOVA, it is observed that, the model for pullulanase and amylase activities were highly significant, as it was evident from the Fisher, F-test and a very low probability value. The *F* values corresponding to pullulanase and amylase was 5.195 and 9.350, respectively and *P* values of the models was less than 0.00007 and 0.000001 for pullulanase and amylase, respectively. Greater the *F* value is from one, the more certain that the factors explain adequately the variation in the data about its mean and the estimated factor effects are real. *P* values were used as a tool to check the significance of each of the coefficient which in turn, was necessary to understand the pattern of the mutual interactions between the test variables. Smaller the magnitude of *P* more significant is the corresponding coefficient (Khuri and Cornell, 1987). Goodness of the model was checked by the coefficient of determination, R^2. Closer the values of R (multiple correlation coefficient) to 1, better the correlation between observed and predicted values. Hence we observed the values of R for pullulanase (0.901) and amylase (0.931) indicate a good agreement between the experimental and predicted yields, respectively. The R^2 for pullulanase and amylase were 0.81 and 0.87, respectively. Coefficient of variation (CV) indicates the degree of precision with which the treatments are compared. Usually, higher the value of CV, lower is the reliability of experiments performed. Here a lower value of CV for pullulanase (7.56) and amylase (7.16) indicates a greater reliability of the experiments performed.

From Table 6 (the significance of regression coefficients of pullulanase activity model), it is observed that the linear terms of maltose were highly significant on pullulanase yields, followed by quadratic terms of maltose , manganous chloride, peptone and bajra flour. None of the calcium chloride terms were significant on pullulanase yield but the interactive terms of any of these test variables on pullulanase activity were found to be significant. Similarly, from Table 7 (the significance of regression coefficients of amylase activity model), it is observed that the linear terms of maltose and quadratic terms of bajra flour were highly significant on amylase yields followed by linear terms of calcium chloride, quadratic terms of maltose and manganous chloride. The interactive terms of maltose and peptone was found significant on amylase activity. However, none of the linear and quadratic terms of peptone were significant on amylase activity.

Interactions among the nutrients

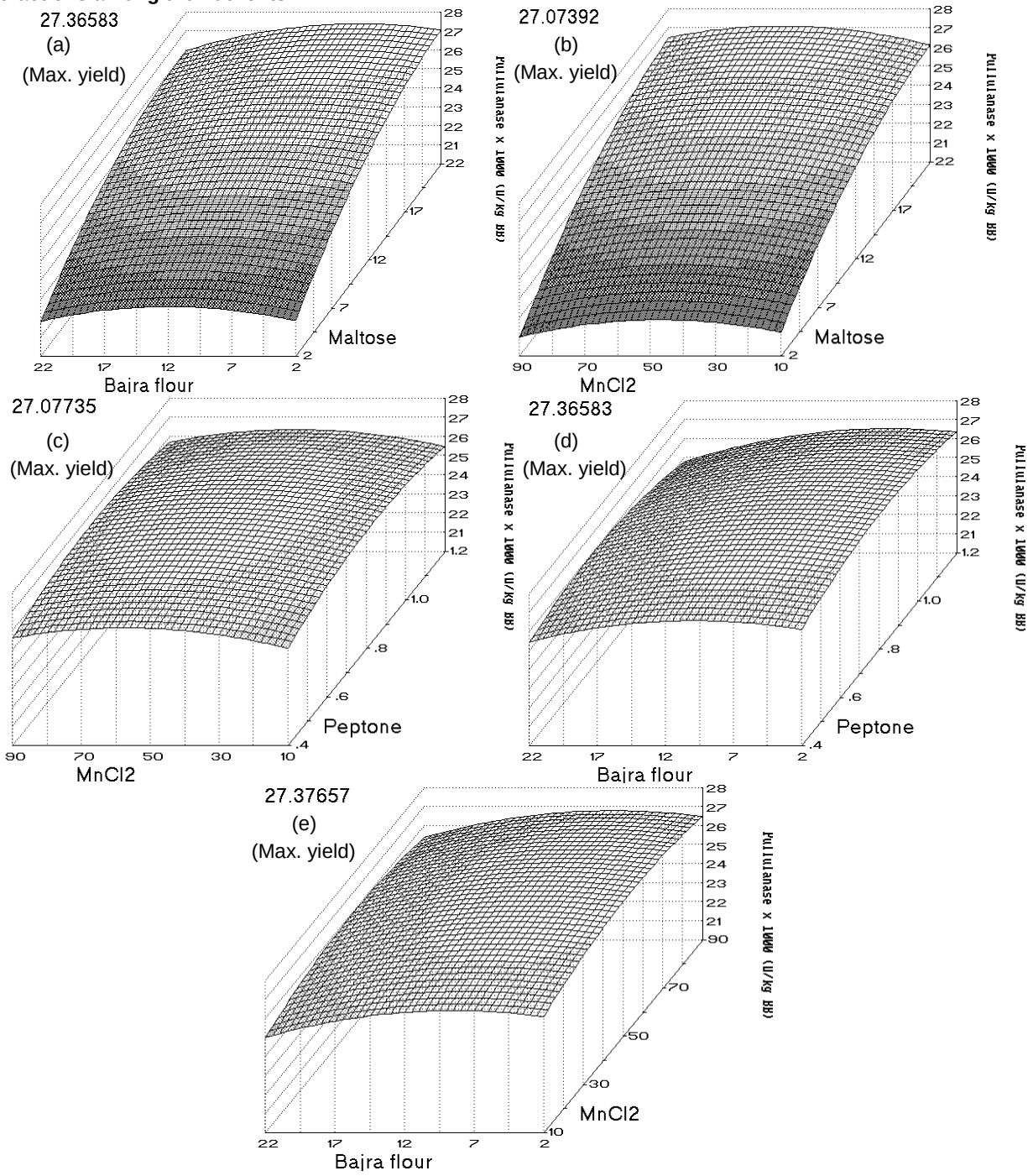

Figure 1: Response surface plot for the production of thermostable amylopullulanase by *C. thermosulfurogenes* SVM17 in SSF as a function of varying concentrations of **(a)** maltose and bajra flour on the yield of pullulanase when the peptone (0.8 % w/w), $MnCl_2 \cdot 4H_2O$ (50.0 ppm w/w) and $CaCl_2 \cdot 2H_2O$ (15.0 ppm w/w) were held at zero level; **(b)** maltose and $MnCl_2 \cdot 4H_2O$ on the yield of pullulanase when the peptone (0.8 % w/w), $CaCl_2 \cdot 2H_2O$ (15.0 ppm w/w) and bajra flour (12.0% w/w) were held at zero level; **(c)** peptone and $MnCl_2 \cdot 4H_2O$ on the yield of pullulanase when the maltose (12.0 % w/w), $CaCl_2 \cdot 2H_2O$ (15.0 ppm w/w) and bajra flour (12.0% w/w) were held at zero level; (d) peptone and bajra flour on the yield of pullulanase when the maltose (12.0 % w/w), $MnCl_2 \cdot 4H_2O$ (50.0 ppm w/w) and $CaCl_2 \cdot 2H_2O$ (15.0 ppm w/w) were held at zero level; (e) $MnCl_2 \cdot 4H_2O$ and bajra flour on the yield of pullulanase when the maltose (12.0 % w/w), peptone (0.8 % w/w) and $CaCl_2 \cdot 2H_2O$ (15.0 ppm w/w) were held at zero level

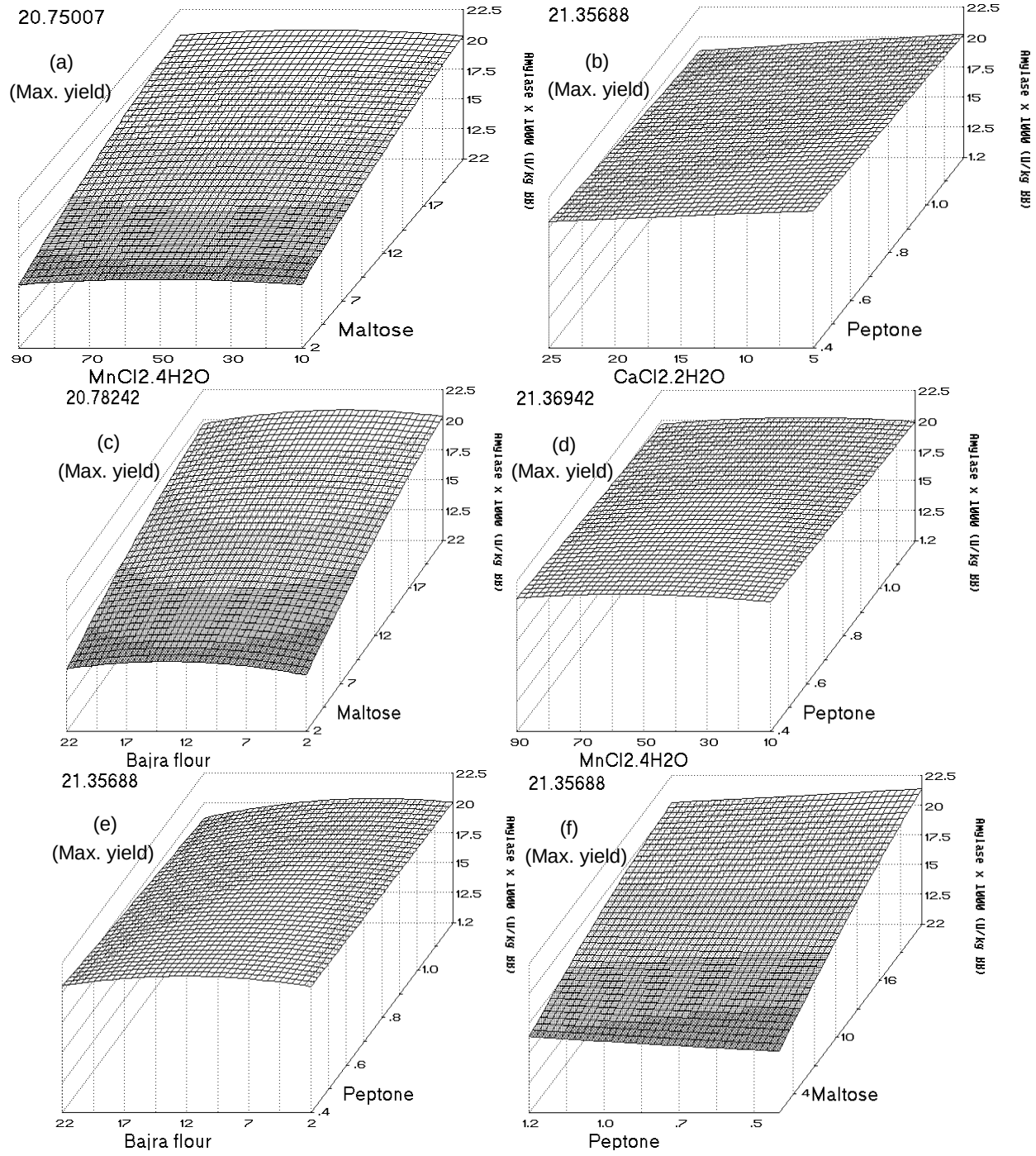

Figure 2: Response surface plot for the production of thermostable amylopullulanase by *C. thermosulfurogenes* SVM17 in SSF as a function of varying concentrations of **(a)** maltose and $MnCl_2 \cdot 4H_2O$ on the yield of amylase when the peptone (0.8 % w/w), $CaCl_2 \cdot 2H_2O$ (15.0 ppm w/w) and bajra flour (12.0% w/w) were held at zero level; **(b**) peptone and $CaCl_2 \cdot 2H_2O$ on the yield of amylase when the maltose (12.0 % w/w), $MnCl_2 \cdot 4H_2O$ (50.0 ppm w/w) and bajra flour (12.0% w/w) were held at zero level; **(c)** maltose and bajra flour on the yield of amylase when the peptone (0.8 % w/w), $MnCl_2 \cdot 4H_2O$ (50.0 ppm w/w) and $CaCl_2 \cdot 2H_2O$ (15.0 ppm w/w) were held at zero level; **(d)** peptone and $MnCl_2 \cdot 4H_2O$ on the yield of amylase when the maltose (12.0 % w/w), $CaCl_2 \cdot 2H_2O$ (15.0 ppm w/w) and bajra flour (12.0% w/w) were held at zero level; **(e)** peptone and bajra flour on the yield of amylase when the maltose (12.0 % w/w), $MnCl_2 \cdot 4H_2O$ (50.0 ppm w/w) and $CaCl_2 \cdot 2H_2O$ (15.0 ppm w/w) were held at zero level; **(f)** maltose and peptone and on the yield of amylase when the $MnCl_2 \cdot 4H_2O$ (50.0 ppm w/w), $CaCl_2 \cdot 2H_2O$ (15.0 ppm w/w) and bajra flour (12.0 % w/w) were held at zero level

Table 7: Significance of regression coefficients of amylase activity (in SSF) model*

Process variable	Regression coefficient	Computed t-value	Probability	Significance level
INTERCEPT	17508.20	25.85	0.0000	
X_1 Maltose	2377.70	12.16	0.0000	* * *
X_2 Peptone	-110.30	-0.56	0.5765	
X_3 $MnCl_2$ $4H_2O$	65.60	0.34	0.7395	
X_4 $CaCl_2$ $2H_2O$	-442.40	-2.26	0.0306	*
X_5 Bajra flour	-142.40	-0.73	0.4716	
X_1^2	-562.60	-2.63	0.0129	*
X_2^2	28.90	0.14	0.8934	
X_3^2	-446.40	-2.09	0.0447	*
X_4^2	-35.10	-0.16	0.8704	
X_5^2	-835.10	-3.91	0.0005	* * *
X_1X_2	-593.30	-2.71	0.0106	*
X_1X_3	10.40	0.05	0.9623	
X_1X_4	-125.20	-0.57	0.5704	
X_1X_5	- 287.70	-1.32	0.1975	
X_2X_3	-137.60	-0.63	0.5335	
X_2X_4	- 125.70	-0.58	0.5692	
X_2X_5	-339.50	-1.55	0.1303	
X_3X_4	63.00	0.29	0.7749	
X_3X_5	1.80	0.01	0.9935	
X_4X_5	-101.30	-0.46	0.6461	
Block	894.30	2.26	0.0311	

* Significance levels of regression coefficients are given as *** 99.9% , ** 99.0% and * 95.0% by t-test.
Regression coefficients, t-values and P values of amylase activity were obtained by subjecting the amylase yields (Table 5) to statistical analysis using 'Indostat' statistical software.

Figures 1a-e and 2a-f are the significant response surface curves for pullulanase and amylase activities of thermostable amylopullulanase, respectively, as a function of concentrations of two medium components with other three components held at zero level. From the response surface plots, it is easy and convenient to understand the interactions between two nutrients and also to locate their optimum levels.

From Figures 1a and 1b, it is observed that the pullulanase activity increased up on increasing the concentrations of maltose from 2–22%. Similarly, the enzyme yield also increased on increasing the concentration of peptone from 0.2–0.8% and any further increase in its concentration, showed gradual decrease in enzyme yield (Figures 1c and 1d). Therefore, 0.8% of peptone was found to be optimum for pullulanase activity. The maximum enzyme yields were recorded when the concentration of manganous chloride increased from 10–50 ppm (Figures 1b, 1c and 1e) and bajra flour 2-10% (Figures 1a, 1d and 1e). On further increase in their concentrations, a gradual decrease in enzyme yields was observed. Therefore, 50 ppm of manganous chloride and 10% bajra flour was considered favorable for maximum yield of pullulanase.

It is clear from response surface plots (Figures 2a, 2c and 2f), that the yield of amylase increased on increasing the concentration of maltose from 2–22%. The increase was more pronounced at lower concentrations of peptone (Figure 2f). Therefore, high levels of maltose and low levels of peptone are favorable for enzyme production. Similarly, maximum amylase activity was observed with increasing concentration of manganous chloride from 10–50 ppm (Figure 2a) and bajra flour from 2–12% (Figure 2c). Further increase in their concentrations resulted in gradual decrease in amylase activity. Therefore, for optimum enzyme yields, 50 ppm of manganous chloride and 12% bajra flour should be considered. The effect of peptone was more significant on amylase yields at higher concentrations of maltose (Figure 2f), at lower levels of calcium chloride (Figure 2b) and moderate levels of manganous chloride (50 ppm) and bajra flour (12%) (Figures 2d and 2e). This indicates that lower levels of calcium chloride, moderate levels of manganous chloride and bajra flour are most favorable for maximum amylase activity at low concentrations of peptone.

From the above observations, it is clear that the maximum pullulanase and amylase activities were observed when the concentrations of test variables lie in the following ranges (%w/w): maltose, 17–22; peptone, 0.4–0.8; manganous chloride, 50 ppm; calcium chloride, 5–15 ppm and bajra flour 7–10.

Based on the above observations, the model predicted (% w/w): maltose, 22; peptone, 0.8; $MnCl_2 \cdot 4H_2O$, 50 ppm; $CaCl_2$, 15 ppm and bajra flour, 11 were required for the maximum production of the enzyme. The low and high levels of $CaCl_2$ decreased the pullulanase and amylase activities, respectively. Keeping in view of both enzyme activities, we have fixed calcium chloride at zero

Table 8: Analysis of variance (ANOVA) table: Effect of medium components on pullulanase activity of amylopullulanase produced by *C. thermosulfurogenes* SVM17 in SSF

Source of variation	Degrees of Freedom	Sum of squares	Mean squares	F-ratio	Probability
Block	1	6986700.00	6986700.00	2.379	0.13508
Model	21	320402250.00	15257250.00	5.195	0.00007
Residual	26	76366580.00	2937180.00		
Total	47	403755540.00	8590540.00		

Root mean square error =1713.82, CV =7.56%, R^2 (coefficient of determination) = 0.81, R =0.901, adjusted R^2 =0.65809

Table 9: Analysis of variance (ANOVA) table: Effect of medium components on amylase activity of amylopullulanase produced by *C. thermosulfurogenes* SVM17 in SSF

Source of variation	Degrees of Freedom	Sum of squares	Mean squares	F-ratio	Probability
Block	1	17994220.00	17994220.00	11.767	0.00168
Model	21	300260820.00	14298130.00	9.350	0.00000
Residual	32	48933160.00	1529160.00		
Total	53	367188190.00	6928080.00		

Root mean square error =1236.59, CV =7.16%, R^2 (coefficient of determination) = 0.87, R =0.931, adjusted R^2 =0.77928

Table 10: Experimental verification of combined effect of optimized medium on the response of amylopullulanase in SSF

Medium components	Range studied (w/w) %	Levels after optimization		Enzyme yield (U/Kg BB)					
		coded	uncoded	Before optimization		After optimization			
				Amylase	Pullulanase	Amylase Predicted	Experimental	Pullulanase Predicted	Experimental
Maltose (X_1)	2.0–22.0	2	22.0						
Peptone (X_2)	0.4–1.2	0	0.8						
$MnCl_2 \cdot 4H_2O$ (X_3)	10–90 ppm	0	50.0	9,220	10,080	21,050	20,130	26,310	28,970
$CaCl_2 \cdot 2H_2O$ (X_4)	5–25 ppm	0	15.0						
Bajra flour (X_5)	2.0–22.0	-0.193	11.035						

level (i.e., 15 ppm, Table 10). By substituting the correspondingly coded concentration levels of the factors in to the regression equation, the maximum predictable response for pullulanase and amylase activities was calculated. The maximum yield of amylase and pullulanase obtained using the optimized medium was 20,130 and 28,970 U/kg BB, respectively. It was in correlation with the predicted yields (Table 10).

There are no reports available in literature on medium optimization for amylopullulanase production by SSF. However, statistical methods have been applied in other studies to optimize the medium components in SSF for the production of cyclosporine-A by *Tolypocladium inflatum* (Ramana Murthy *et al*., 1999), alkaline exo-polygalactouronase by *Bacillus subtilis* RCK (Gupta *et al*., 2008), lipase by *Candida rugosa* (Venkat Rao *et al*., 1993), thermostable pullulanase by *Clostridium thermosulfurogenes* SV2 (Rama Mohan Reddy *et al*., 1999a), xylanase by *Thermascus aurantiacus* (Souza de O *et al*., 1999). In all these cases the product yields were higher in SSF than in SmF. Increase in the yield of cyclosporin-A from 459–4843 mg/kg was achieved in SSF by using statistical method for optimization of medium (Ramana Murthy, *et al*., 1999). Similarly, yield of gibberelic acid and lipase activity were increased in about 11 and 3 folds, respectively (Pastrana *et al*., 1995; Venkat Rao *et al*., 1993). After medium optimization, the increase in pullulanase production by *C. thermosulfurogenes* SV2 was observed to be increased by 10% (Rama Mohan Reddy *et al*., 1999a). Therefore these studies indicate the feasibility study of SSF processes.

CONCLUSIONS

In the present study screening of nutrients employing Plackett-Burman design and optimization of nutrients concentration using response surface methodology for the production of thermostable amylopullulanase in SSF proved to be useful in increasing the yields of pullulanase and amylase by 182 and 106 %, respectively in a limited number of experiments. Hence the application of statistical experimental designs for production of

thermostable amylopullulanase was found to be more promising as these designs are relatively simple and time saving. An overall increase in yield of pullulanase and amylase was found to be 22 and 8 folds, respectively in SSF after optimization when compared to SmF. Therefore SSF process is highly advantageous for production of valuable thermostable amylopullulanase over SmF process.

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