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Optimization of medium components using response surface methodology for production of thermostable amylopullulanase in submerged fermentation by *Clostridium thermosulfurogenes* SVM17

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ABSTRACT

Response surface methodology (RSM) based on central composite rotatable design (CCRD) was used to determine the optimal levels of medium components, *viz*., soluble starch, tapioca flour, peptone, magnesium chloride and ferrous sulphate for enhanced thermostable amylopullulanase production by *Clostridium thermosulfurogenes* SVM17 in submerged fermentation. The design contains 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 the central points. Within the tested range of concentrations, all medium components were found significant. The optimum levels of nutrients for maximum production of enzyme were (% w/v): potato starch, 5.2; tapioca flour, 6.3; peptone, 2.5; $MgCl_2 \cdot 6H_2O$, 0.015 and $FeSO_4 \cdot 7H_2O$, 6.0 ppm. After optimization of medium components, the strain SVM17 showed 96 and 409 % increased amylase and pullulanase activities, respectively when compared with the non-optimized conditions.

Keywords: Amylase, pullulanase, submerged fermentation, response surface methodology, *Clostridium thermosulfurogenes*

INTRODUCTION

Bioprocessing of starch into maltose and maltooligosaccharides by enzymatic means is gaining importance, because of their potential application in food, pharmaceutical, beverage and fine chemical industries (Fogarty and Kelly, 1990; Saha *et al*., 2009). They are produced by hydrolysis of starch using amylases from higher plants, certain mesophilic bacteria and fungi. The majority of amylases reported so far are optimally active at moderate temperatures (Haki and Rakshit, 2003). Therefore a high value is placed on extreme thermostable enzymes. Presence of both α-1,4- and α-1,6- hydrolyzing enzymes will have added advantages. Thermoanaerobes show promise for production of thermostable enzymes, and efforts have been made to isolate thermoanaerobic bacteria that produce an enzyme which can hydrolyze both α-1,6- linkages in pullulan and starch and α-1,4- linkages in starch. Since the enzymes have both amylase and pullulanase activities, the term amylopullulanase has been coined to categorize them (Saha and Zeikus, 1989; Spreinat and Antranikian, 1990; Kwak *et al*., 1998; Zareian *et al*., 2010; Mrudula *et al*., 2011a; 2011b). Earlier, we have isolated anaerobic, thermophilic and amylolytic bacteria in our laboratory (Swamy and Seenayya, 1996a). The strains were further screened for production of maltose and maltooligosaccharides and the strain *Clostridium thermosulfurogenes* SVM17, which produced increased yield of amylopullulanase and stable at 100 °C was selected (Mrudula, 2010; Mrudula *et al.*, 2010; Mrudula *et al*., 2011a; 2011b).

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 yields of the product, reduced process variability, closer confirmation of the out put response to normal and target requirements, reduced development time with overall costs (Mrudula, 2010). 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 (Naidu and Panda, 1999; Jagannadha Rao *et al*., 2000; Teng and Xu, 2008; Satyanarayana and Pradeep, 2009). This method is a time consuming process and requires a number of experiments to determine optimum levels, 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, 1951; Khuri and Cornell, 1987). RSM has gained importance for optimization of media components and parameters (Uma

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Maheswara Rao and Satyanarayana, 2007; Teng and Xu, 2008; Reddy *et al*., 2008; Mrudula *et al.*, 2010; Mrudula *et al.,* 2011b). To the best of our knowledge there is no report on optimization of nutrient levels for amylopullulanase using RSM in SmF. In the present study, RSM based on central composite rotatable design (CCRD) was applied to determine the optimum concentrations of medium components for increased production of thermostable amylopullulanase by *C. thermosulfurogenes* SVM17 in SmF.

MATERIALS AND METHODS

Microorganism and culture conditions

The bacterial strain used in the present study was isolated in our laboratory and identified as *Clostridium thermosulfurogenes* SVM17 (Swamy and Seenayya, 1996a; Mrudula, 2010; Mrudula *et al*., 2011a; Mrudula *et al.,* 2011b). The organism was cultivated anaerobically in 120 mL serum vials that contained 20 mL of peptone yeast extract (PYE) medium (Swamy and Seenayya, 1996b).

Submerged fermentation medium and experimental parameters

Basal medium (20 mL) containing (g/L) KH_2PO_4, 0.3; $Na_2HPO_4{\cdot}7H_2O$, 2.0 and resazurin, 0.002 dissolved in distilled water were dispensed into 120 mL serum vials. Five medium components, *viz*., soluble starch (0.5-6.5%), tapioca flour (0.5-6.5%), peptone (1.5-3.5%), $MgCl_2{\cdot}6H_2O$ (0.005-0.025%) and $FeSO_4{\cdot}7H_2O$ (2.0-10.0 ppm) with 3.5, 3.5, 2.5, 0.015 and 6.0 ppm, respectively as their central points under study were added to each vial as given in RSM experimental design (Table 1). The concentration range for each nutrient was fixed based on the literature (Rama Mohan Reddy *et al*., 2003) and on our own experience gained. The medium was flushed with nitrogen gas to create anaerobic conditions and vials were sealed and sterilized at 121 °C for 15 min. The vials were cooled to r.t. (28 ± 2 °C and 2% (v/v) of 2.5% (w/v) Na_2S was added, pH was adjusted to 7.5, inoculated with 5% (v/v) exponentially grown culture and incubated at 60 °C for 24 h.

Experimental design

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; Rama Mohan Reddy *et al*., 2003; Mrudula, 2010), 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 quadratic coefficients and $b_{12\ -15}$, $b_{23\text{-}25}$, $b_{34\text{-}35}$ and b_{45} are the interactive coefficients.

Significance of the model was 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; Mrudula 2010). 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 software (Indostat services, Hyderabad, India; Anonymous, 1998). Optimum concentration of each nutrient is identified based on the hump in three dimensional plots.

Enzyme assay

Amylase and pullulanase activities were measured by incubating 0.5 ml of appropriately diluted enzyme source with 0.5 ml of 1% (w/v) starch solution and pullulan solution, respectively in 2 mL of 0.1 M acetate buffer (pH 5.5) at 70 °C for 30 min respectively. After incubation, 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 of formation of (1 U mol) of reducing sugars (as glucose equivalents) per min under standard assay conditions.

RESULTS AND DISCUSSION

C. thermosulfurogenes SVM17 grew optimally at growth temperature of 60 °C, at an initial pH of 7.5 and incubation period of 24 h. Under these conditions, the strain produced 2600 and 1300 U of thermostable amylase and pullulanase activities, respectively per litre of culture broth

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using PYE medium containing 0.5% (w/v) soluble starch as carbon source.

Previously 15 nutrients comprising of four each of carbon, nitrogen, minerals and then complex organic sources (flours) were screened using Plackett-Burman design for thermostable amylopullulanase production by *C. thermosulfurogens* SVM17 in SmF (Mrudula *et al*., 2010). Among them five medium components, *viz*., starch, tapioca flour, peptone, $MgCl_2$ and $FeSO_4$ have been identified as most significant and further selected for optimization of their levels using RSM in SmF.

Table 1: 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 SmF.

Combination number	Soluble starch % (w/v) X_1	Tapioca flour % (w/v) X_2	Peptone % (w/v) X_3	$MgCl_2.6H_2O$ % (w/v) X_4	$FeSO_4.7H_2O$ ppm X_5
1	-1 (2.0)	-1 (2.0)	-1 (2.0)	-1 (0.01)	-1 (4.0)
2	1 (5.0)	-1 (2.0)	-1 (2.0)	-1 (0.01)	-1 (4.0)
3	-1 (2.0)	1 (5.0)	-1 (2.0)	-1 (0.01)	-1 (4.0)
4	1 (5.0)	1 (5.0)	-1 (2.0)	-1 (0.01)	-1 (4.0)
5	-1 (2.0)	-1 (2.0)	1 (3.0)	-1 (0.01)	-1 (4.0)
6	1 (5.0)	-1 (2.0)	1 (3.0)	-1 (0.01)	-1 (4.0)
7	-1 (2.0)	1 (5.0)	1 (3.0)	-1 (0.01)	-1 (4.0)
8	1 (5.0)	1 (5.0)	1 (3.0)	-1 (0.01)	-1 (4.0)
9	-1 (2.0)	-1 (2.0)	-1 (2.0)	1 (0.02)	-1 (4.0)
10	1 (5.0)	-1 (2.0)	-1 (2.0)	1 (0.02)	-1 (4.0)
11	-1 (2.0)	1 (5.0)	-1 (2.0)	1 (0.02)	-1 (4.0)
12	1 (5.0)	1 (5.0)	-1 (2.0)	1 (0.02)	-1 (4.0)
13	-1 (2.0)	-1 (2.0)	1 (3.0)	1 (0.02)	-1 (4.0)
14	1 (5.0)	-1 (2.0)	1 (3.0)	1 (0.02)	-1 (4.0)
15	-1 (2.0)	1 (5.0)	1 (3.0)	1 (0.02)	-1 (4.0)
16	1 (5.0)	1 (5.0)	1 (3.0)	1 (0.02)	-1 (4.0)
17	-1 (2.0)	-1 (2.0)	-1 (2.0)	-1 (0.01)	1 (8.0)
18	1 (5.0)	-1 (2.0)	-1 (2.0)	-1 (0.01)	1 (8.0)
19	-1 (2.0)	1 (5.0)	-1 (2.0)	-1 (0.01)	1 (8.0)
20	1 (5.0)	1 (5.0)	-1 (2.0)	-1 (0.01)	1 (8.0)
21	-1 (2.0)	-1 (2.0)	1 (3.0)	-1 (0.01)	1 (8.0)
22	1 (5.0)	-1 (2.0)	1 (3.0)	-1 (0.01)	1 (8.0)
23	-1 (2.0)	1 (5.0)	1 (3.0)	-1 (0.01)	1 (8.0)
24	1 (5.0)	1 (5.0)	1 (3.0)	-1 (0.01)	1 (8.0)
25	-1 (2.0)	-1 (2.0)	-1 (2.0)	1 (0.02)	1 (8.0)
26	1 (5.0)	-1 (2.0)	-1 (2.0)	1 (0.02)	1 (8.0)
27	-1 (2.0)	1 (5.0)	-1 (2.0)	1 (0.02)	1 (8.0)
28	1 (5.0)	1 (5.0)	-1 (2.0)	1 (0.02)	1 (8.0)
29	-1 (2.0)	-1 (2.0)	1 (3.0)	1 (0.02)	1 (8.0)
30	1 (5.0)	-1 (2.0)	1 (3.0)	1 (0.02)	1 (8.0)
31	-1 (2.0)	1 (5.0)	1 (3.0)	1 (0.02)	1 (8.0)
32	1 (5.0)	1 (5.0)	1 (3.0)	1 (0.02)	1 (8.0)
33	0 (3.5)	0 (3.5)	0 (2.5)	0 (0.015)	0 (6.0)
34	0 (3.5)	0 (3.5)	0 (2.5)	0 (0.015)	0 (6.0)
35	0 (3.5)	0 (3.5)	0 (2.5)	0 (0.015)	0 (6.0)
36	0 (3.5)	0 (3.5)	0 (2.5)	0 (0.015)	0 (6.0)
37	0 (3.5)	0 (3.5)	0 (2.5)	0 (0.015)	0 (6.0)
38	0 (3.5)	0 (3.5)	0 (2.5)	0 (0.015)	0 (6.0)
39	0 (3.5)	0 (3.5)	0 (2.5)	0 (0.015)	0 (6.0)
40	0 (3.5)	0 (3.5)	0 (2.5)	0 (0.015)	0 (6.0)
41	-2 (0.5)	0 (3.5)	0 (2.5)	0 (0.015)	0 (6.0)
42	2 (6.5)	0 (3.5)	0 (2.5)	0 (0.015)	0 (6.0)
43	0 (3.5)	-2 (0.5)	0 (2.5)	0 (0.015)	0 (6.0)
44	0 (3.5)	2 (6.5)	0 (2.5)	0 (0.015)	0 (6.0)

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45	0 (3.5)	0 (3.5)	-2 (1.5)	0 (0.015)	0 (6.0)
46	0 (3.5)	0 (3.5)	2 (3.5)	0 (0.015)	0 (6.0)
47	0 (3.5)	0 (3.5)	0 (2.5)	-2 (0.005)	0 (6.0)
48	0 (3.5)	0 (3.5)	0 (2.5)	2 (0.025)	0 (6.0)
49	0 (3.5)	0 (3.5)	0 (2.5)	0 (0.015)	-2 (2.0)
50	0 (3.5)	0 (3.5)	0 (2.5)	0 (0.015)	2 (10.0)
51	0 (3.5)	0 (3.5)	0 (2.5)	0 (0.015)	0 (6.0)
52	0 (3.5)	0 (3.5)	0 (2.5)	0 (0.015)	0 (6.0)
53	0 (3.5)	0 (3.5)	0 (2.5)	0 (0.015)	0 (6.0)
54	0 (3.5)	0 (3.5)	0 (2.5)	0 (0.015)	0 (6.0)

uncoded units are given in parentheses

Response surface analysis for the optimization of nutrient levels

The predicted and experimental yields of pullulanase and amylase for 54 experiments are given in Table 2. The regression coefficients and *t*-values of pullulanase and amylase are given in Tables 3 and 4, respectively. Analysis of variance (ANOVA) is required to test the significance and adequacy of the model. From Tables 3 and 4, ANOVA of the model demonstrates that the model is highly significant, as is evident from Fisher, *F*-test (F model, mean square regression/mean square residual) and a very low probability value. The F values corresponding to pullulanase and amylase are 4.702 and 4.457, respectively and *P* values of the models were less than 0.00005 and 0.00008 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 co-efficient of determination, R^2. Closer the values of R (multiple correlation coefficients) to 1, better the correlation between observed and predicted values. Hence we observed the values of R for pullulanase (0.870) and amylase (0.864) indicate a good agreement between the experimental and predicted values of pullulanase and amylase yields, respectively. The R^2 for pullulanase and amylase were 0.757 and 0.746, 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 (9.56 for pullulanase and 9.92 for amylase) indicates a greater reliability of the experiments performed.

The regression coefficients and corresponding *P* values (Tables 3 and 4) suggest that, among the test variables, linear relationship between soluble starch and enzyme yields and tapioca flour and enzyme yields were highly significant followed by both linear and quadratic relations between peptone and enzyme yields. Ferrous sulphate also influences the enzyme yields but its quadratic effect was more pronounced than the linear effect. The mutual interaction between tapioca flour and magnesium chloride is also important and other interactions are insignificant.

Interactions among the nutrients

Figures 1a,b to 5a,b are the significant response surface curves for amylase and pullulanase 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, b**,** it can be seen that the yields of both amylase and pullulanase were increased as the concentration of soluble starch increased from 0.5 to 6.5%. Similarly the enzyme yields were increased up on increasing the concentrations of tapioca flour from 0.5 to 5.0% (Figures 2a, b) at higher concentrations of peptone (Figures 2a**,** b). High concentration of magnesium chloride is required for maximum production of enzymes in the presence of high concentrations of peptone (2.5-3.0%) (Figures 4a,b) and ferrous sulphate (8.0 ppm) (Figures 5a,b). High enzyme yields of amylase and pullulanase were recorded when ferrous sulphate was used at a concentration of 8.0 ppm (Figures 3 a, b).

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/v): soluble starch 5-6.5; tapioca flour, 5.0; peptone, 2.5-3.0; ferrous sulphate, 7.0-8.0 ppm and magnesium chloride, 0.015-0.02. Based on the above observations, the model predicted optimum levels of nutrients for maximum production of enzyme were (% w/v): potato starch, 5.2; tapioca flour, 6.3; peptone, 2.5; magnesium chloride, 0.015 and ferrous sulphate, 6.0 ppm. By substituting the corresponding coded concentration levels of factors into the regression equation, maximum predictable response for amylase and pullulanase activities were calculated. Maximum yield of amylase and pullulanase obtained using the optimized medium was 5,099 and 6,610 U/L, respectively. It was in correlation with the predicted yields. After optimization, the yields of amylase and pullulanase were increased by 96 and 409 %, respectively.

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Table 2: Experimental and predicted pullulanase and amylase activities of thermostable amylopullulanase produced by *C. thermosulfurogenes* SVM17 in submerged fermentation.

C.No*	Pullulanase activity (U/L)			Amylase activity (U/L)		
	Experimental yield	Predicted yield	Residual	Experimental yield	Predicted yield	Residual
1	3,663	3,654	9	3,019	2,995	24
2	5,167	4,970	198	4,341	4,159	182
3	5,269	4,499	770	4,316	3,661	655
4	5,644	5,526	118	4,740	4,605	135
5	4,338	4,373	-35	3,552	3,621	-69
6	5,413	5,311	102	4,547	4,550	-3
7	4,628	4,762	-134	3,791	3,885	-94
8	4,912	5,412	-500	4,126	4,594	-468
9	3,480	3,582	-102	2,923	2,996	-73
10	3,995	4,536	-541	3,356	3,802	-446
11	5,185	5,107	78	4,246	4,283	-37
12	6,321	5,773	548	5,334	4,869	465
13	4,971	4,450	521	4,071	3,658	413
14	5,623	5,027	596	4,723	4,229	494
15	5,080	5,519	-439	4,160	4,543	-383
16	6,041	5,809	233	5,227	4,894	333
17	4,082	3,897	185	3,342	3,284	58
18	5,802	5,307	495	4,873	4,419	454
19	3,207	4,414	-1207	2,693	3,727	-1034
20	5,227	5,536	-309	4,390	4,642	-252
21	4,463	5,066	-603	3,655	4,205	-550
22	5,679	6,100	-421	4,848	5,105	-257
23	5,993	5,128	865	5,011	4,246	765
24	6,123	5,872	251	5,143	4,926	217
25	3,871	3,513	358	3,432	3,101	331
26	4,441	4,563	-122	3,730	3,878	-148
27	5,020	4,711	309	4,537	4,165	372
28	5,570	5,472	98	4,884	4,722	162
29	4,569	4,832	-263	3,838	4,059	-221
30	5,352	5,505	-153	4,495	4,601	-106
31	5,328	5,574	-246	4,475	4,720	-245
32	5,600	5,958	-358	4,704	5,043	-339
33	5,826	5,775	51	4,893	4,862	32
34	5,882	5,775	107	4,940	4,862	79
35	6,187	5,775	412	5,198	4,862	337
36	5,453	5,775	-322	4,580	4,862	-282
37	5,338	5,775	-437	4,484	4,862	-338
38	5,515	5,775	-260	4,633	4,862	-229
39	5,862	5,775	87	4,925	4,862	64
40	5,834	5,775	59	4,900	4,862	39
41	4,391	4,386	5	3,688	3,602	86
42	6,006	6,086	-80	4,918	5,088	-170
43	4,779	4,853	-74	4,014	4,014	0
44	6,151	6,152	-1	5,037	5,122	-85
45	3,588	3,993	-405	3,015	3,398	-383
46	5,527	5,197	330	4,642	4,344	298
47	5,408	5,263	145	4,429	4,269	160
48	5,056	5,276	-220	4,141	4,386	-245
49	4,193	4,866	-673	3,522	4,045	-523
50	5,857	5,259	598	4,920	4,482	438

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51	5,878	5,759	119	4,937	4,810	127
52	5,785	5,759	26	4,859	4,810	49
53	5,849	5,759	90	4,913	4,810	103
54	5,898	5,759	139	4,954	4,810	144

*Combination number

Experiments were conducted in 120 mL serum vials with respective concentrations of nutrients as per the design (Table 1) along with KH_2PO_4 (0.3 g/L) and Na_2HPO_4 (2.0 g/L) dissolved in distilled water incubated at 60 °C for 24 h. The enzyme activities were assayed under standard assay conditions.

Table 3: Significance of regression coefficients of pullulanase activity (in SmF) model[a].

Process variable	Regression coefficient	Computed t-value	Significance level
INTERCEPT	5790.38	21.30	
X_1 soluble starch	424.83	5.41	* * *
X_2 tapioca flour	324.58	4.13	* * *
X_3 peptone	301.18	3.83	* * *
X_4 $MgCl_2$. $6H_2O$	3.33	0.04	
X_5 $FeSO_4$. $7H_2O$	98.13	1.25	
X_1^2	-130.71	-1.52	
X_2^2	-64.09	-0.75	
X_3^2	-290.96	-3.39	* * *
X_4^2	-122.34	-1.43	
X_5^2	-174.09	-2.03	*
X_1X_2	-72.09	-0.82	
X_1X_3	-94.28	-1.07	
X_1X_4	-90.16	-1.02	
X_1X_5	23.72	0.27	
X_2X_3	-113.90	-1.30	
X_2X_4	170.22	1.94	*
X_2X_5	-81.91	-0.93	
X_3X_4	37.28	0.43	
X_3X_5	112.65	1.2	
X_4X_5	-77.72	-0.89	
Block	-15.77	-0.10	

[a]: Significance levels of regression coefficients are given as *** 99.9%, ** 99.0% and * 95.0 by t-test: "*F* ratio" for the model was 4.702 (degrees of freedom were 21, 32). Probably = 0.00005, R^2 = 0.7573 and R^2 = adjusted = 0.59804

Table 4: Significance of regression coefficients of amylase activity (in SmF) model[a].

Process variable	Regression coefficient	Computed t-value	Significance level
INTERCEPT	4913.40	20.78	
X_1 soluble starch	371.50	5.44	* * *
X_2 tapioca flour	276.95	4.06	* * *
X_3 peptone	236.60	3.47	* * *
X_4 $MgCl_2$. $6H_2O$	29.30	0.43	
X_5 $FeSO_4$. $7H_2O$	109.35	1.60	
X_1^2	-116.07	-1.56	
X_2^2	-60.44	-0.81	
X_3^2	-234.69	-3.15	* * *
X_4^2	-120.57	-1.62	
X_5^2	-136.57	-1.83	*
X_1X_2	-55.06	-0.72	
X_1X_3	-58.75	-0.77	
X_1X_4	-89.31	-1.17	
X_1X_5	-7.25	-0.09	
X_2X_3	-100.50	-1.32	
X_2X_4	155.19	2.03	*
X_2X_5	-55.75	-0.73	
X_3X_4	9.13	0.12	
X_3X_5	73.94	0.97	
X_4X_5	-45.89	-0.60	
Block	-51.85	-0.38	

[a]: Significance levels of regression coefficients are given as *** 99.9%, ** 99.0% and * 95.0 by t-test: "*F* ratio" for the model was 4.457degrees of freedom were 21, 32). Probably = 0.00008, R^2 = 0.7573 and R^2 = adjusted = 0.57989

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Several reports are available in literature on statistical approaches (such as RSM) for optimization of medium components and various physical parameters for production of different microbial products. In SmF, the RSM has been applied for production of α-amylase by *Bacillus* sp. (Tanyildizi *et al*., 2005), *Geobacillus thermoleovorans* (Uma Maheswara Rao and Satyanarayana, 2007), lipase by *Geotrichum* sp. (Burkert *et al*., 2004), whole cell lipase by *Rhizopus chinensis* (Teng and Xu, 2008), phytase by *E. coli* (Sunitha *et al*., 1999), glucosyl transferase by *Aspergillus niger* (Lee and Chen, 1997), laccase by *Botryosphaeria* sp. (Vasconcelos *et al*., 2000). A two fold increase in α-amylase production was reported in *B. circulans* GRS313 (Dey *et al*., 2002) by use of response surface methodology. Rama Mohan Reddy *et al*., (1999; 2003) reported about 10 and 36% more pullulanase and β-amylase by *Clostridium thermosulfurogens* SV2 after optimization of medium composition. Amylopullulanase of *Clostridium thermosulfurogens* SVM 17 showed increase in the yields of amylase and pullulanase by 106 % and 182 %, respectively in SSF (Mrudula, 2010).

Kapat *et al*. (1998) used RSM to evaluate optimum environmental conditions for production of glucose oxidase. With these optimized physical parameters, glucose oxidase production by recombinant *S. cerevisiae* was increased by 74 % compared to un-optimized conditions.

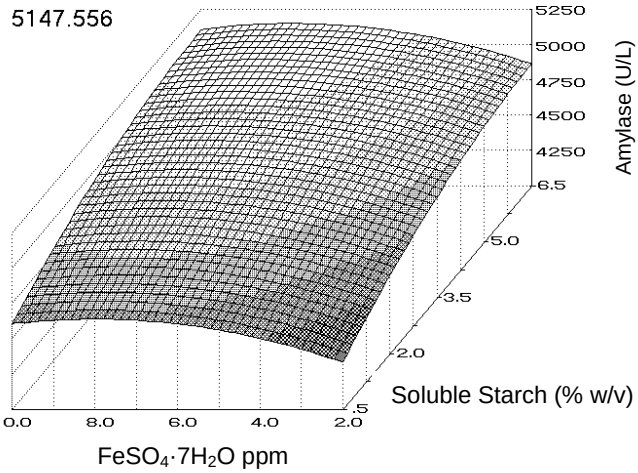

Figure 1a: Response surface plot for the production of thermostable amylopullulanase by *C. thermosulfurogenes* SVM 17 in SmF as a function of varying concentrations of soluble starch and $FeSO_4 .7H_2O$ on the yield of amylase when the tapioca flour (3.5 % w/v), peptone (2.5 % w/v) and $MgCl_2.6H_2O$ (0.015 % w/v) were held at zero level.

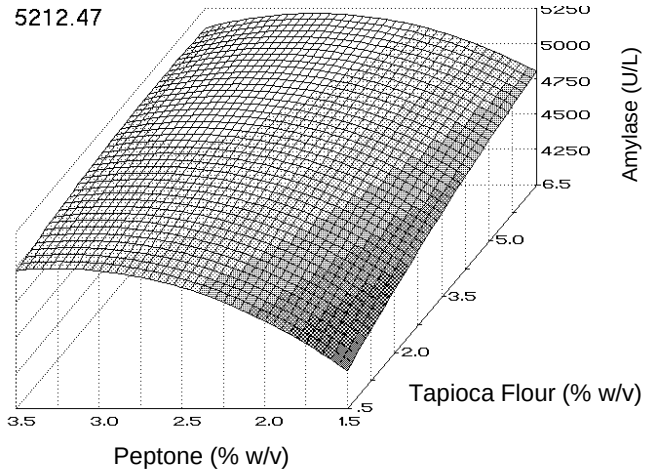

Figure 2a: Response surface plot for the production of thermostable amylopullulanase by *C. thermosulfurogenes* SVM 17 in SmF as a function of varying concentrations of tapioca flour and peptone on the yield of amylase when the soluble starch (3.5% w/v), $MgCl_2.6H_2O$ (0.015% w/v) and $FeSO_4·7H_2O$ (6.0 ppm w/v) were held at zero level.

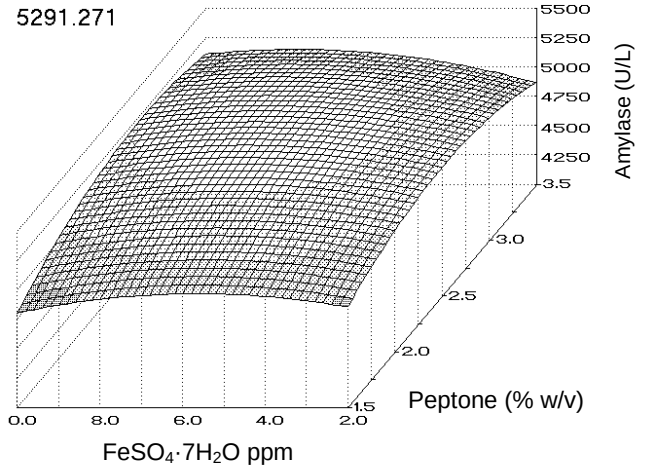

Figure 3a: Response surface plot for the production of thermostable amylopullulanase by *C. thermosulfurogenes* SVM17 in SmF as a function of varying concentrations of peptone and $FeSO_4·7H_2O$ on the yield of amylase when the soluble starch (3.5% w/v), tapioca flour (3.5% w/v) and $MgCl_2·6H_2O$ (0.015% w/v) were held at zero level.

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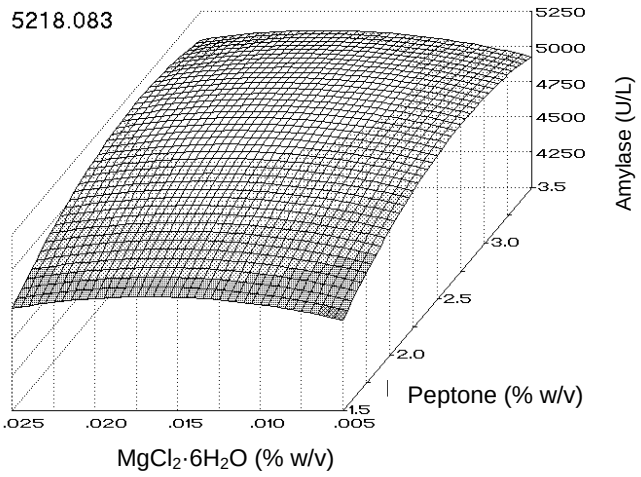

Figure 4a: Response surface plot for the production of thermostable amylopullulanaseby *C. thermosulfurogenes* SVM17 in SmF as a function of varying concentrations of peptone and $MgCl_2 \cdot 6H_2O$ on the yield of amylase when the soluble starch (3.5% w/v), tapioca flour (3.5% w/v) and $FeSO_4 \cdot 7H_2O$ (6.0 ppm w/v) were held at zero level.

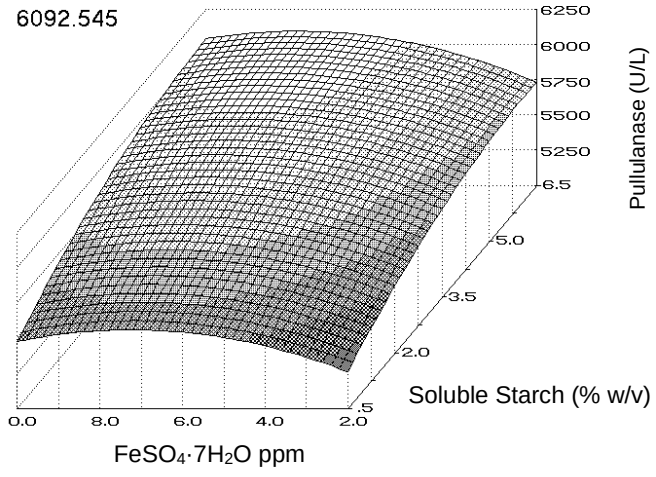

Figure 1b: Response surface plot for the production of thermostable amylopullulanase by *C. thermosulfurogenes* SVM 17 in SmF as a function of varying concentrations of soluble starch and $FeSO_4 \cdot 7H_2O$ on the yield of pullulanase when the tapioca flour (3.5% w/v), peptone (2.5% w/v) and $MgCl_2 \cdot 6H_2O$ (0.015% w/v) were held at zero level.

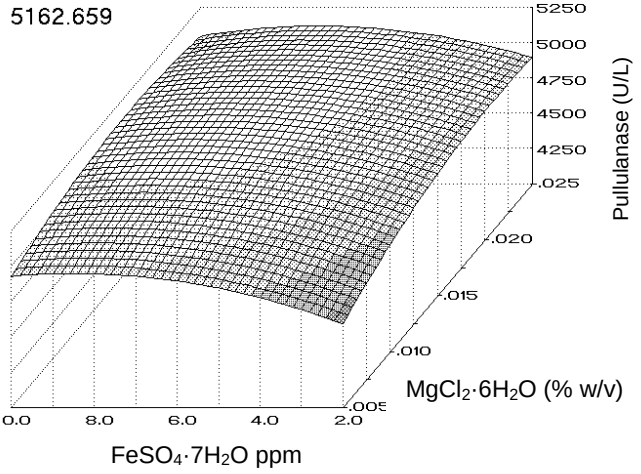

Figure 5a: Response surface plot for the production of thermostable amylopullulanase by *C. thermosulfurogenes* SVM17 in SmF as a function of varying concentrations of $MgCl_2 \cdot 6H_2O$ and $FeSO_4 \cdot 7H_2O$ on the yield of amylase when the soluble starch (3.5% w/v), tapioca flour (3.5% w/v) and peptone (2.5% w/v) were held at zero level.

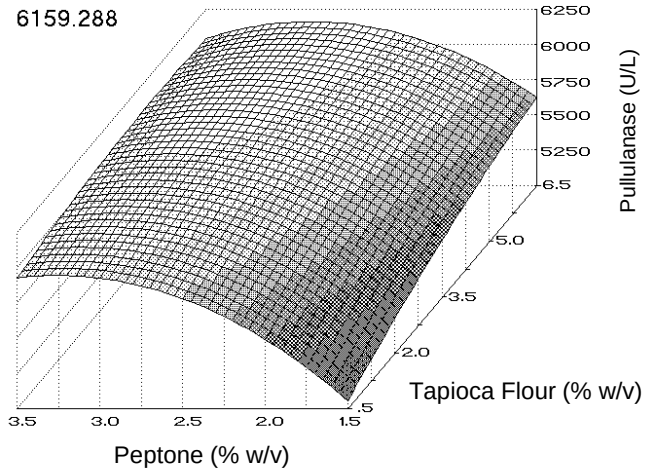

Figure 2b: Response surface plot for the production of thermostable amylopullulanase by *C. thermosulfurogenes* SVM 17 in SmF as a function of varying concentrations of tapioca flour and peptone on the yield of pullulanase when the soluble starch (3.5% w/v), $MgCl_2 \cdot 6H_2O$ (0.015% w/v) and $FeSO_4 \cdot 7H_2O$ (6.0 ppm w/v) were held at zero level.

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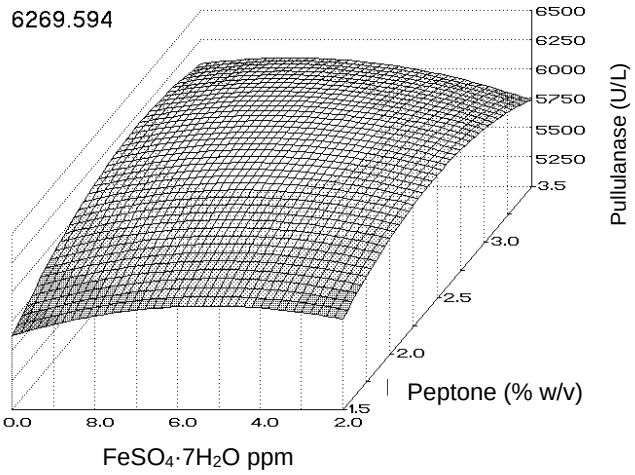

Figure 3b: Response surface plot for the production of thermostable amylopullulanase by *C. thermosulfurogenes* SVM17 in SmF as a function of varying concentrations of peptone and $FeSO_4 \cdot 7H_2O$ on the yield of pullulanase when the soluble starch (3.5% w/v), tapioca flour (3.5% w/v) and $MgCl_2 \cdot 6H_2O$ (0.015% w/v) were held at zero level.

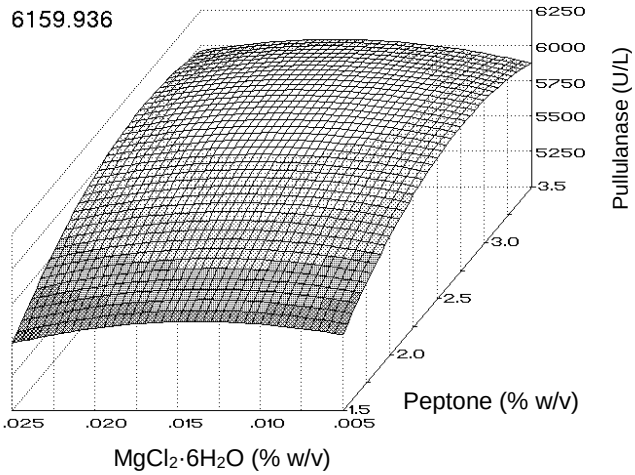

Figure 4b: Response surface plot for the production of thermostable amylopullulanase by *C. thermosulfurogenes* SVM17 in SmF as a function of varying concentrations of peptone and $MgCl_2 \cdot 6H_2O$ on the yield of pullulanase when the soluble starch (3.5% w/v), tapioca flour (3.5% w/v) and $FeSO_4 \cdot 7H_2O$ (6.0 ppm w/v) were held at zero level.

CONCLUSIONS

Conventional medium formulation studies are usually time consuming and expensive (Srinivas *et al*., 1994; Rama Mohan Reddy *et al*., 1999). To overcome these problems, an efficient fermentation medium for production of thermostable amylopullulanase with increased yields by *C. thermosulfurogenes* SVM17 has been developed. RSM was used to optimize the medium components. The optimized medium produced 96 and 409% more of

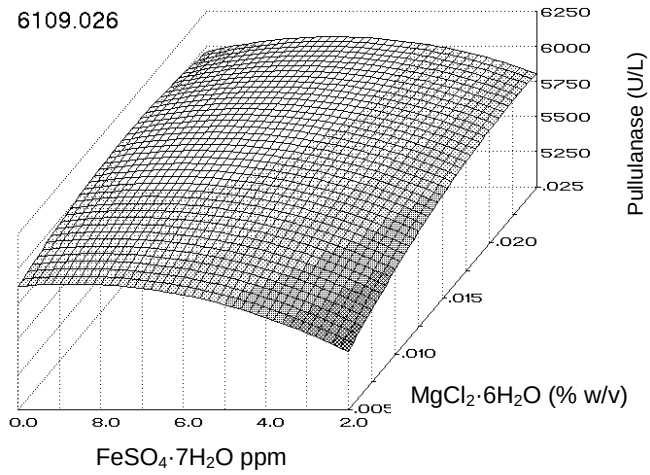

Figure 5b: Response surface plot for the production of thermostable amylopullulanase by *C. thermosulfurogenes* SVM17 in SmF as a function of varying concentrations of $MgCl_2 \cdot 6H_2O$ and $FeSO_4 \cdot 7H_2O$ on the yield of pullulanase when the soluble starch (3.5% w/v), tapioca flour (3.5% w/v) and peptone (2.5% w/v) were held at zero level.

thermostable amylase and pullulanase, respectively which means that the productivity was greatly increased: The utilization of raw material with low cost, for example tapioca flour reduces overall cost of fermentation medium for amylopullulanase production therefore amylopullulanase production by *C. thermosulfurogenes* SVM17 can be considered as highly promising for industrial production than the un-optimized medium.

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