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Production of thermostable β-amylase and pullulanase by *Clostridium thermosulfurogenes* SV2 in solid-state fermentation: Screening of nutrients using Plackett-Burman design

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Abstract Screening of fifteen nutrients belonging to four categories, viz., carbon, nitrogen, salt and complex organic sources was carried out using Plackett-Burman design for the production of thermostable β-amylase and pullulanase by *Clostridium thermosulfurogenes* SV2 in solid-state fermentation (SSF). This design involves screening of up to '$n-1$' variables in just 'n' number of experiments. Regression co-efficients and t-values were calculated by subjecting the experimental data to statistical analysis. Lactose, diammonium hydrogen phosphate, calcium chloride and casein hydrolysate showed higher regression co-efficients in the biomass formation. Among the fifteen nutrients screened, based on their performance in terms of product promoting ability, availability and cost, magnesium chloride, potato starch, ferrous sulphate, pearl millet flour and corn steep liquor were identified as most effective and, therefore, selected for inclusion in further optimization studies.

1 Introduction

Maltose and maltooligosaccharides have applications in food, beverage and pharmaceutical industries. They are produced by the hydrolysis of starch using amylases from higher plants such as barley, sweet potato, soybean and wheat and also from certain mesophilic bacteria. So far reported β-amylases and pullulanases are thermally unstable and are very expensive [1, 2, 3, 4]. A high value is placed on thermostable and thermoactive amylases 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 [5]. Efforts have been made to isolate thermoanaerobic bacteria that produce thermostable β-amylase [1, 3, 6, 7] and pullulanase [2, 8, 9, 10, 11]. In this direction, we have isolated an anaerobic, thermophilic and amylolytic bacterium, *Clostridium thermosulfurogenes* SV2 that produces high yields of both thermostable β-amylase and pullulanase [12]. We have purified these enzymes to homogeneity and characterized [13] and studied their production in submerged [14] and solid-state fermentation [15]. In recent times, the bacterial systems are increasingly investigated for the production of enzymes and metabolites by solid-state fermentation (SSF) [16]. The SSF has numerous advantages over submerged fermentation (SmF), including superior productivity, simple technique, low capital investment, low energy requirement and less water output, better product recovery and lack of foam build up [17] and reported to be the most appropriate process for developing countries [18].

Selection of appropriate carbon, nitrogen and other nutrients is one of the most critical stages in the development of an efficient and economic process. The methodologies used for screening the nutrients fall into two major categories; classical and statistical [19]. The statistical methodologies are preferred because of various advantages in their use in terms of rapid and reliable short-listing of nutrients, understanding the interactions among the nutrients at varying concentrations and a tremendous reduction in total number of experiments resulting in saving of time, glassware, chemicals and manpower [20, 21]. Plackett-Burman design is a statistical methodology that is used for 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. In spite of the above advantages, the statistical designs are applied to a limited number of submerged fermentation processes [22], aerobic SSF processes [20, 23] and never attempted earlier in anaerobic submerged or SSF processes. In the present study, we report the screening of nutrients using Plackett-Burman design for the production of thermostable β-amylase and pullulanase by *C. thermosulfurogenes* SV2 in SSF.

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 [12] using TYE medium [24].

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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. N_2 was used as head space gas. 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 [25] followed by centrifugation (8000 × *g* for 20 min) and the supernatant was used as enzymes source.

2.3
Screening of nutrients using Plackett-Burman design

Fifteen nutrients belonging to four categories, viz., carbon sources (lactose, potato starch, maltose and dextrin), nitrogen sources (peptone, corn steep liquor, casein hydrolysate and diammonium hydrogen phosphate), complex organic sources (wheat flour, potato flour and pearl millet flour) and salt/mineral sources (ferrous sulphate, magnesium chloride, calcium chloride and potassium chloride) were screened using the Plackett-Burman design (Table 1). The concentration for each nutrient was fixed based on the literature and on our own experience gained. Carbon and other nutrient sources, except for those mentioned below, 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. Disaccharide sugars and corn steep liquor were prepared as 10X solutions and separately autoclaved at 10 lbs for 10 min. Diammonium hydrogen phosphate was sterilized in dry form by exposing to germicidal UV light (Philips, 30 Watts, 2733 A) for 30 min [20]. Just before inoculations, a 2% (v/w) of 2.5% (w/v) Na_2S 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%.

2.4
Biomass estimation

The DNA method was followed for the estimation of biomass. The sample (100 ml) containing the stationary phase-grown culture was centrifuged (8000 × *g*) for 20 min at 4 °C, the pellet was washed twice with 1 mM TE buffer (pH 8.0) and recentrifuged. The pellet was suspended in 5 ml TE buffer (pH 8) and the cells were lysed by sonication. The sonicated sample was centrifuged (8000 × *g*) for 20 min at 4 °C and the DNA content in the supernatant and pellet, after adding equal volume of 10% trichloroacetic acid to each, was determined by DPA method [26]. The method was calibrated using the DNA content of the known amount of *C. thermosulfurogenes* SV2 biomass obtained in submerged culture. The method was corrected for the DNA content of the medium.

2.5
Enzyme assays

The β-amylase and pullulanase activities in the clarified samples were measured by incubating 0.5 ml appropriately diluted enzymes source with 0.5 ml of 2% (w/v) starch and 1% (w/v) pullulan, respectively, at their optimum temperatures (70 °C for β-amylase and 75 °C for pullulanase) in 2 ml of phosphate buffer (0.1M, pH 6.0). Reducing sugars released were measured by a 3, 5 dinitrosalycilic acid method [27]. A separate blank was set

Table 1. Plackett-Burman design for screening of fifteen nutrients along with their ranges for the production of thermostable β-amylase and pullulanase in SSF

Ingredient	Concentration of ingredient		Combinations															
	'–' value (% w/w)	'+' value (% w/w)	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
Lactose	3	6	+	+	+	+	−	+	−	+	+	−	−	+	−	−	−	−
Potato starch	3	6	−	+	+	+	+	−	+	−	+	+	−	−	+	−	−	−
Maltose	3	6	−	−	+	+	+	+	−	+	−	+	+	−	−	+	−	−
Dextrin	3	6	−	−	−	+	+	+	+	−	+	−	+	+	−	−	+	−
Peptone	0.4	0.8	+	−	−	−	+	+	+	+	−	+	−	+	+	−	−	−
Corn steep liquor	0.4	0.8	−	+	−	−	−	+	+	+	+	−	+	−	+	+	−	−
Casein hydrolysate	0.4	0.8	−	−	+	−	−	−	+	+	+	+	−	+	−	+	+	−
$(NH_4)_2HPO_4$	1.64	3.28	+	−	−	+	−	−	−	+	+	+	+	−	+	−	+	−
Wheat flour	3	6	+	+	−	−	+	−	−	−	+	+	+	+	−	+	−	−
Potato flour	3	6	−	+	+	−	−	+	−	−	−	+	+	+	+	−	+	−
Pearl millet flour	3	6	+	−	+	+	−	−	+	−	−	−	+	+	+	+	−	−
$FeSO_4 \cdot 7H_2O$	10 ppm	20 ppm	−	+	−	+	+	−	−	+	−	−	−	+	+	+	+	−
$MgCl_2 \cdot 6H_2O$	0.02	0.04	+	−	+	−	+	+	−	−	+	−	−	−	+	+	+	−
$CaCl_2 \cdot H_2O$	10 ppm	20 ppm	+	+	−	+	−	+	+	−	−	+	−	−	−	+	+	−
KCl	0.05	0.10	+	+	+	−	+	−	+	+	−	−	+	−	−	−	+	−

'+' and '–' levels indicate the higher and lower levels, respectively, of an ingredient in that combination

up for each sample to correct the non-enzymatic release of sugars. One unit of β-amylase or pullulanase was defined as the amount of enzyme which released 1 μmol of reducing sugars as glucose min^{-1} under the standard assay conditions.

2.6 Statistical analysis of the data

The regression co-efficients and *t*-values were calculated by compatible analysis [28, 29] of the data on the yields of thermostable β-amylase and pullulanase obtained in the experiments. The 'Indostat' statistical package was used for the data analysis. The ingredient with highest *t*-value is considered as the best nutrient [29] and thus selected for further optimization studies.

3 Results and discussion

C. thermosulfurogenes SV2 grew optimally at 60 °C and produced 770 and 910 U of thermostable β-amylase and pullulanase, respectively, per litre culture broth in submerged fermentation [13, 14]. The strain SV2 produced on an average 1022 and 1142 U of thermostable β-amylase and pullulanase, respectively, per kilogram BB when grown at 60 °C in 24 hr on wheat bran that was moistened with distilled water.

3.1 Effect of nutrients on growth and enzymes production

In the present study, *C. thermosulfurogenes* SV2 grew optimally and produced highest average yields of β-amylase (2434 U/kg BB) and pullulanase (3948 U/kg BB) in combination 13 of the design followed by in combinations 14, 2 and 5 (β-amylase) and 1, 14 and 7 (pullulanase) in that order (Table 2).

The results of the data analysis obtained from the growth, β-amylase and pullulanase produced by *C. thermosulfurogenes* SV2 in screening of fifteen nutrients through Plackett-Burman design are presented in Table 3. It is apparent from the data that potato starch, pearl millet flour, $MgCl_2 \cdot 6H_2O$, $FeSO_4 \cdot 7H_2O$, $CaCl_2 \cdot H_2O$, corn steep liquor and peptone showed comparatively greater positive effects on the production of both the enzymes. Diammonium hydrogen phosphate and casein hydrolysate showed positive effect on pullulanase production and potato flour showed positive effect on β-amylase production. Among the carbon sources screened, potato starch was found to be the best for the enzyme production. All the four carbon sources supported the growth of the organism with lactose having the maximum effect. Potato starch and maltose had almost similar effect on the growth but the former had highest positive effect on the enzyme yields. This suggests that the potato starch had the inducing effect on the enzyme synthesis. In SmF, potato starch was reported as the best substrate for β-amylase production by *Bacillus polymyxa* No. 72 [30] and for pullulanase production by *C. thermosulfurogenes* SV9 [11] and *B. cereus* isolate [31]. In another study, starch and dextrin were inducers and maltose was repressor for the production of amylase by *B. stearothermophilus* [32]. In contrast, maltose was reported to be the inducer of β-amylase in *C. thermosulfurogenes* [1] and *C. thermocellum* SS8 [7]. Corn steep liquor was found to have highest positive effect on both the enzymes yield. The positive effect of corn steep liquor on the growth and enzymes production could be due to the presence of growth factors in it [20]. Swamy & Seenayya [11] reported that corn steep liquor in combination with yeast extract was the best nitrogen source for thermostable α-amylase and pullulanase production in SmF by *C. thermosulfurogenes* SV9. No information was available on the production of thermostable β-amylase and pullulanase in SSF.

Earlier, the SSF has been employed for the production of thermostable α-amylase by *Bacillus licheniformis* [33,

Table 2. Yields of thermostable β-amylase and pullulanase by *C. thermosulfurogenes* SV2 obtained in screening of fifteen nutrients using Plackett-Burman design

Combination[a]	Biomass (g/kg BB)		β-Amylase (U/Kg BB)		Pullulanase (U/Kg BB)	
	Set I	Set II	Set I	Set II	Set I	Set II
1	5.81	5.86	1,198	1,264	3,261	3,216
2	5.98	5.84	1,892	1,804	2,885	2,791
3	5.65	5.73	1,648	1,541	2,685	2,621
4	5.41	5.38	1,042	1,121	2,581	2,611
5	5.92	5.98	1,863	1,761	2,091	2,138
6	5.71	5.82	1,702	1,754	1,810	1,858
7	5.79	5.58	1,328	1,311	2,939	2,881
8	3.44	3.59	768	712	2,738	2,694
9	4.12	4.05	794	861	2,707	2,755
10	6.02	5.87	1,811	1,886	2,910	2,844
11	5.31	5.38	1,091	1,007	1,998	1,942
12	5.58	5.41	1,118	1,195	2,281	2,206
13	6.28	6.17	2,420	2,448	3,901	3,994
14	6.09	6.14	1,967	1,912	3,061	3,191
15	5.71	5.84	1,398	1,309	2,708	2,605
16	5.34	5.42	1,602	1,664	2,692	2,815

[a]See table 1 for the combinations

Experiments were conducted in 10 g wheat bran that contained respective nutrients with an initial moisture level of 65%, an initial pH of 7.2 and incubated at 60 °C for 24 h

Table 3. Regression co-efficients and *t*-values calculated from the β-amylase and pullulanase yields obtained in the screening experiments

Ingredient	Biomass		β-amylase		Pullulanase	
	Reg. co-eff	*t*-value	Reg. co-eff	*t*-value	Reg. co-eff	*t*-value
INTERCEPT	5.53	172.88	1,474.75	161.87	2,717.18	176.17
Lactose	−0.32	10.20	−198.87	−21.82	−110.93	−7.19
Potato starch	0.07	2.26	120.93	13.27	116.18	7.53
Maltose	−0.07	2.28	−0.62	−0.07	−197.62	−12.81
Dextrin	−0.10	3.16	−183.81	−20.17	−335.25	−21.73
Peptone	0.01	0.43	58.93	6.47	17.93	1.16
Corn steep liquor	−0.20	6.47	10.93	1.20	75.62	4.90
Casein hydrolysate	−0.25	7.80	−127.31	−13.97	55.68	3.61
$(NH_4)_2HPO_4$	−0.27	8.52	−154.12	−16.91	124.37	8.06
Wheat flour	0.04	1.46	−10.75	−1.18	−41.12	−2.66
Potato flour	0.23	7.19	151.75	16.65	−89.75	−5.82
Pearl millet flour	0.18	5.77	0.93	0.10	152.12	9.86
$FeSO_4 \cdot 7H_2O$	0.01	0.29	70.87	7.78	96.31	6.24
$MgCl_2 \cdot 6H_2O$	0.14	4.42	140.25	15.39	104.18	6.75
$CaCl_2 \cdot H_2O$	0.26	8.27	68.93	7.56	76.06	4.93
KCl	−0.07	2.34	−106.31	−11.67	−80.12	−5.19

34] and *B. megaterium* [35], α-amylase by *B. coagulans* [17], proteases [23], alpha-galactosidase [20], tannin acyl hydrolase [36] and pectinase [37] by *Aspergillus niger* and L-glutaminase by *Vibrio costicola* [38]. The enzyme yields in SSF, in general, are reported to be many folds more than in the SmF [16, 20, 23]. The cost of production of amyloglucosidase [39] and α-amylase [25] in SSF was very less when compared to their production in SmF.

From the present study, it is evident that the use of Plackett-Burman design not only helped us in short-listing few nutrients, but also proved to be useful in increasing the yields of β-amylase and pullulanase by 137 and 245%, respectively, in a limited number of experiments. Overall, based on the product promoting ability, availability, cost and the need to keep the number of factors as low as possible for optimization studies using response surface methodology, only five nutrients, viz., $MgCl_2 \cdot 6H_2O$, potato starch, $FeSO_4 \cdot 7H_2O$, pearl millet flour and corn steep liquor, have been identified as most effective. Further, studies are in progress to optimize the concentrations of these selected nutrients using response surface methodology for the production of thermostable β-amylase and pullulanase by *C. thermosulfurogenes* SV2 in SSF.

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