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STATISTICAL OPTIMIZATION OF PROCESS PARAMETERS FOR POLYGALACTURONASE PRODUCTION BY *Bacillus subtilis* KP10B

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ABSTRACT

In present study the statistical optimization of process parameters for cost-effective production of polygalacturonase (PGase) by *Bacillus subtilis* was examined by using Plackett-Burman design (PBD) and central composite design (CCD). Of the 9 factors, temperature, $CaCO_3$ and KH_2PO_4 were found significant by PBD and were further optimized by CCD. After optimization, maximal PGase activity (272.87 Um L^{-1}) was achieved at 40°C, 3.5 mM concentration of KH_2PO_4 and 6.5 mM concentration of $CaCO_3$. The results showed 2.95-fold increase in PGase activity of *B. subtilis* under optimized conditions. Thus, it may be concluded that optimized medium is cost-effective and suitable for maximum production and may be used for large-scale PGase production by *B. subtilis* KP10B. The crude enzyme extracts of *B. subtilis* can be used in industry for fruit juice clarification, waste water treatment and coffee fermentation.

Keywords: *Bacillus subtilis*, central composite design, Plackett-Burman design, polygalacturonase, statistical optimization

INTRODUCTION

Polygalacturonase (PGase) (pectinase, EC 3.2.1.15) is an enzyme that produces oligomers of D-galacturonic acid by breaking α-1,4-glycosidic link between galacturonic acid residues (Patidar *et al*., 2017). PGase shares 25% of the global food enzyme sales and has broad range of applications, including extraction and clarification of fruit juices, scouring of cotton, degumming of plant fibers, treatment of wastewater, extraction of vegetable oil, fermentation of tea and coffee, paper bleaching, additive for poultry feed, and alcoholic beverages (Patidar *et al*., 2017; Shrestha *et al*., 2021). Agro-wastes serve as valuable energy and nutrient rich raw material, therefore can be used as an alternate source for the production of industrially important enzymes, which otherwise would be waste if shredded in open dump yards and landfills (Kavuthodi and Sebastian, 2018). Wheat bran is considered most effective of all the substrates (Oumer and Abate, 2018). Namasivayam *et al*. (2011) observed that wheat bran increased pectinase production by *B. cereus,* isolated from market solid waste.

One of the most perilous stages in the development of efficient and cost-effective bioprocess is its optimization process (Kavuthodi and Sebastian, 2018). In production of industrially important enzymes, a variety of factors like medium composition, ambient conditions, and strain variations are investigated for optimum yield (Nawawi *et al*., 2022). As the demand of pectinase is increasing continuously, the hefty production cost, however, continues to be a stumbling block. As a result, the industries attempt to develop more cost-effective production by using agricultural, vegetable and

fruit waste. Another strategy is to optimize the fermentation conditions and nutritional compositions in order to enhance organism development and enzyme production (Ahmed *et al.*, 2021). The traditional strategy for optimizing the enzyme production by one variable at a time entails altering a single independent variable while keeping the others fixed. This one-dimensional approach is tedious and time-consuming, especially while dealing with a large number of variables and does not take into account the variable interactions (Sharma and Satyanarayana, 2006). Response surface methodology (RSM) entails conducting a full factorial search by investigating all components simultaneously in a systematic and efficient manner (Sharma and Satyanarayana, 2006). The present study was aimed to identify vital factors affecting the PGase production and to statistically optimize them for maximizing cost effective PGase production from *Bacillus subtilis* KP10B strain.

MATERIALS AND METHODS

Chemicals and reagents

Pectin and others chemicals used in present study were of analytical grade and purchased from Sigma-Aldrich Chemicals, USA, Microbiological media and Hi-media Laboratories, Mumbai, India. Agro-residues were collected locally (Aurangabad and Paithan, India).

Microorganisms

Bacillus subtilis KP10B, isolated from soil, was used as a source for PGase production. The culture was maintained on nutrient agar slants at 4°C. The isolate was identified on the basis of 16S rRNA gene sequencing and the sequence was deposited in GenBank under Accession No. MW585606.

Enzyme assay

PGase activity was assayed by measuring the amount of reducing sugar (galacturonic acid) released by the degradation of polygalacturonic acid using the 3,5, dinitrosalicylic acid method (Miller, 1959). One unit of PG activity (U) was defined as the quantity of enzyme that releases 1 mol of galacturonic acid per minute under assay conditions.

Experimental design and optimization by using RSM for enhanced PGase production

Plackett-Burman design (PBD): The PBD was used to screen the significant factors controlling the PGase production by *B. subtilis* KP10B using Minitab (Release 19, PA, USA) statistical package (Mohandas *et al.*, 2018). All the experiments were carried out in 100 mL flasks. Based on preliminary experiments (one variable at a time optimization), nine media components ($CaCO_3$, casein, wheat bran, KH_2PO_4, pH, $CaCl_2$, temperature, incubation time and inoculum size) that could potentially affect PGase production of the isolate were first screened with a PBD using 12 experimental runs at two levels i.e. minimum [low level (-1)] and maximum [high level (+1)]. Based on one at a time approach, the upper and lower limits of each variable were decided. All the experiments were carried out in triplicate and the average values of pectinase activity were taken as the response. The factors and levels tested were wheat bran (0.5 and 1.5% levels), casein (0.25 and 0.5%), $CaCO_3$ (7 and 10 mM), $CaCl_2$ (3 and 5 mM), KH_2PO_4 (5 and 7 mM), temperature (30 and 40°C), pH (6 and 7), innoculum size (3 and 6%) and incubation time (48 and 72 h).

Central composite design (CCD): Three independent factors found significant on PBD basis were further optimized for PGase production by CCD using Design Expert Software (version 9.0.4) (Gupta *et al.*, 2016). Each factor was studied at three different levels low, medium and high (-1, 0 and +1, respectively) to analyze the optimum level and combined effect of variables. The effect and regression coefficients of individual linear, quadratic, and interaction variables were calculated using analysis of variance (ANOVA) tables created from CCD data. The effects of various factor interactions on PGase production were studied by drawing three-dimensional response curves against any two independent variables while leaving the other independent variables at '0' levels.

Validation of the model

The accuracy of predicted statistical model was validated for the production of PGase under the conditions predicted by the model (Uzuner and Cekmecelioglu, 2015). The experiments were performed in triplicates and the results were compared.

Comparison of PGase production in optimized and unoptimized media

The PGase production produced in optimized media was compared with the previously used unoptimized PGase production media by KP10B.

RESULTS AND DISCUSSION

Plackett-Burman (PB) analysis

A total of 12 experimental combinations were obtained using PBD for 9 independent factors (Table 1) which exhibited PGase activity ranging from 47.43 ±5.52 to 267.52 ± 5.70 U mL^{-1}. With the highest activity of 267.52 ± 5.70 U mL^{-1}, run 11 was judged to be the best acceptable combination. This variation ensured that all of the parameters had an effect on enzyme activity. PB analysis was used to construct a Pareto-Chart (Fig. 1), and it was evident that temperature (40°C), concentration of KH_2PO_4 and $CaCO_3$ had significant effect on PGase production and their P value was < 0.05. Table 2 (coded coefficients) revealed that the P value of temperature was 0.001 and it had positive effect of 87.44, thus indicating that higher temperature influences PGase production. Sharma and Satyanarayana (2012) have reported enhanced pectinase production at 40°C. In general, the optimal temperature for PGase production corresponded to the temperature at which the corresponding microbe grew (Yadav *et al*., 2015). Temperature regulates extracellular enzyme secretion, potentially through modifying the physical properties of cell membrane (Rahman *et al.*, 2005). P value of KH_2PO_4 and $CaCO_3$ were 0.014 and 0.013, respectively, but both showed negative effect of -50.2 and -51.1, respectively, which indicated that KH_2PO_4 and $CaCO_3$ at their lower concentration enhanced the PGase production. The effect of $CaCO_3$ on pectinase production has been extensively reported. $CaCO_3$ may act as a pH regulator in the medium, suppressing enzyme production at acidic pH. The $CaCO_3$ neutralizes the acid and protects from a sudden drop in pH (Spanos and Koutsoukos, 1998; Kavuthodi and Sebastian, 2018). Narayanan *et al.* (2014) reported enhanced PGase production with KH_2PO_4. Sharma and Satyanarayana (2006) working on *Bacillus pumilus* dcsr 1 revealed that pectinase production was enhanced by using KH_2PO_4.

Table 1: Plackett-Burman design for nine independent factors affecting PGase production

Run order	A	B	C	D	E	F	G	H	J	Responses (Enzyme activity in U mL^{-1})	Predicted values
1	1	-1	1	-1	-1	-1	1	1	1	99.58 ± 7.15	103.11
2	1	1	-1	1	-1	-1	-1	1	1	105.10 ± 2.48	154.21
3	-1	1	1	-1	1	-1	-1	-1	1	72.13 ± 4.41	52.91
4	1	-1	1	1	-1	1	-1	-1	-1	176.06 ± 1.99	190.55
5	1	1	-1	1	1	-1	1	-1	-1	97.54 ± 6.45	104.01
6	1	1	1	-1	1	1	-1	1	-1	148.75 ± 6.71	140.34
7	-1	1	1	1	-1	1	1	-1	1	186.43 ± 5.66	190.55
8	-1	-1	1	1	1	-1	1	1	-1	47.43 ± 5.52	52.91
9	-1	-1	-1	1	1	1	-1	1	1	193.38 ± 5.66	191.44
10	1	-1	-1	-1	1	1	1	-1	1	173.84 ± 5.98	191.44
11	-1	1	-1	-1	-1	1	1	1	-1	267.52 ± 5.70	241.64
12	-1	-1	-1	-1	-1	-1	-1	-1	-1	199.59 ± 2.75	154.21

A - Wheat bran, B - Casein, C - $CaCO_3$, D - $CaCl_2$, E - KH_2PO_4, F - Temperature, G – pH of medium, H - Inoculum size, and J - Incubation time.

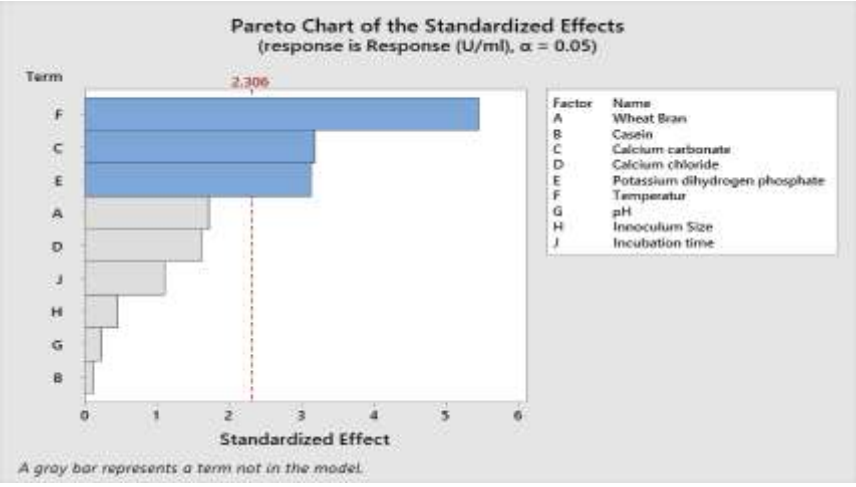

Fig. 1: Effect of different factors on PGase production

Table 2: The coefficient value of 3 significant factors obtained from Placket-Burman analysis

Terms	Effect	P-value
Constant		0.000
C	-51.10	0.013
E	-50.20	0.014
F	87.44	0.001

C = Calcium carbonate,
E = Potassium dihydrogen phosphate
F = Temperature

Central composite design analysis

For three significant factors (temperature, KH_2PO_4 and $CaCO_3$), CCD resulted in a total of 20 experimental combinations (Table 3). The data obtained from CCD was used to create ANOVA (Table 4), which revealed that the lack of fit was non-significant and that the experimental data was extremely reliable. The model F-value of 19.9 implies the model was significant. P-values can be used to determine the significance of model terms. Significant model terms have P-values < 0.05 (Tepe and Dursun, 2021). The quadratic effect of temperature (A^2) was more significant than the quadratic effect of concentration of $CaCO_3$ (B^2) and KH_2PO_4 (C^2), since its P-value was < 0.05, i.e. (P <0.0001). The linear effect of temperature (A), concentration of $CaCO_3$ (B) and KH_2PO_4 (C) and the interaction between temperature and concentration of $CaCO_3$ (AB), concentration of $CaCO_3$ and KH_2PO_4 (BC) and temperature and KH_2PO_4 (AC) were not significant for PGase production. The

Table 3: CCD design for three significant factors affecting PGase production

Run	Factor 1 A: Temperature (˚C)	Factor 2 B: $CaCO_3$ (mM)	Factor 3 C: KH_2PO_4 (mM)	Response (enzyme activity in U mL^{-1})
1	45	10.0	5.0	144.56
2	45	3.0	2.0	154.50
3	40	6.5	3.5	180.34
4	45	10.0	2.0	148.09
5	40	6.5	3.5	189.00
6	35	10.0	2.0	160.10
7	35	10.0	5.0	148.00
8	31.59	6.5	3.5	66.48
9	35	3.0	2.0	159.08
10	40	6.5	3.5	198.90
11	40	0.61	3.5	166.00
12	40	6.5	3.5	189.90
13	40	6.5	3.5	195.60
14	40	6.5	6.02	159.10
15	48.41	6.5	3.5	89.05
16	35	3.0	5.0	140.80
17	40	12.38	3.5	175.77
18	40	6.5	3.5	204.91
19	40	6.5	0.97	181.89
20	45	3.0	5.0	189.60

R^2 value (indicated goodness of fit of model) for a good statistical model should possess minimum value of 0.80 (Bibi *et al*., 2016). The R^2 value obtained from data analysis was 0.9471, suggesting that the model was good. Bibi *et al*. (2016) and Kavuthodi and Sebastian (2018) found R^2 value of 0.9826 and 0.9929, respectively, suggesting a good fit.

The 3D response surface plots give a graphical representation of regression equation that is used to determine the best levels of each parameter for achieving maximum PGase activity. The plot revealed that PGase activity increased with increase in temperature and concentration of $CaCO_3$ and was observed highest (198.9 U mL^{-1}) (middle levels of all three factors) at 40°C with 6.5 mM $CaCO_3$. Similar results have been reported by El Enshasy *et al*. (2018) who studied bioprocess optimization for pectinase production using *Aspergillus niger* in a submerged cultivation system. They observed that pectinase activity was maximum at middle levels of pectin, $(NH_4)_2SO_4$ and K_2HPO_4, and that enzyme levels decreased significantly at higher levels (+ 1).

Table 4: Analysis of model by using ANOVA test for PGase production

Source	Sum of squares	df	Mean square	F-value	p-value	
Model	21620.59	9	2402.29	19.90	< 0.0001	Significant
A (temperature)	20.44	1	20.44	0.169	0.6894	
B ($CaCO_3$)	39.35	1	39.35	0.326	0.5807	
C (KH_2PO_4)	555.73	1	555.73	4.60	0.0575	
AB	11.74	1	11.74	0.097	0.7616	
AC	17.91	1	17.91	0.148	0.7082	
BC	38.59	1	38.59	0.319	0.5843	
A^2	20881.93	1	20881.93	172.96	< 0.0001	
B^2	381.16	1	381.16	3.16	0.1060	
C^2	401.87	1	401.87	3.33	0.0981	
Residual	1207.32	10	120.73	-	-	
Lack of fit	838.08	5	167.62	2.27	0.1946	Non- significant
Pure error	369.23	5	73.85	-	-	
*Cor total	22827.91	19	-	-	-	

*Cor total = Amount of variation around the mean of observations

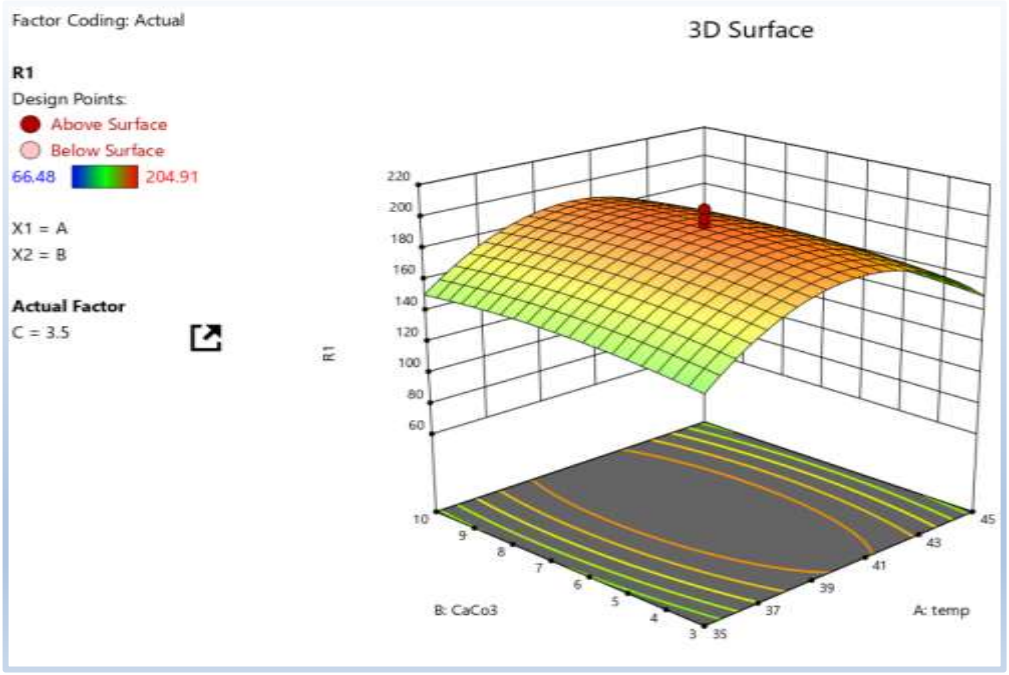

Fig. 2: 3D response surface plot showing the interaction effect of $CaCO_3$ and temperature on PGase activity

Validation of the experiment

Experiments were conducted under optimum conditions as determined by the regression model (temperature 40°C, KH_2PO_4 3.5 mM, and $CaCO_3$ 6.5 mM, wheat bran 0.5 g, casein 0.5 g in 100 mL distilled water) to ensure that the experimental model was validated. The actual response (192.63 U mL^{-1}) predicted by regression model was very near to the PGase activity (272.87 U mL^{-1}) observed in experiments, which proved the validity of model and also indicated a good agreement between the experimental and predicted values. The results showed that the optimization of process parameters through PBD and CCD supported the enhanced PGase production by KP10B isolate. Wong *et al*. (2017) observed similar results when they validated the model and found that the experimental value of polygalacturonase activity was 564.5 U g^{-1}, which was 0.5% higher than the predicted value (561.7 U g^{-1}).

Comparison of PGase production in optimized medium with unoptimized medium

In comparison to the previous pectinase production media examined (99.79 ± 6.99 U mL^{-1}), the optimized medium (280.60 ± 7.07 U mL^{-1}) showed 2.95-fold increase in PGase production. Several studies have been reported enhanced pectinase production after statistical optimization except the enhanced fold in particular study. After optimization from response surface methodology, Kuvvet *et al*. (2019) reported 2-fold increase in pectinase production from *Bacillus* spp by using apple pomace as a carbon source. Under optimized conditions, Uzuner and Cekmecelioglu (2015) obtained 2.7-fold increase in pectinase activity from *B. subtilis*.

Conclusion: BD and CCD were used to enhance PGase production by *B. subtilis* KP10B by 2.95-fold. The overall findings show that the production of PGase by *B. subtilis* KP10B using wheat bran as an effective substrate significantly reduces the medium production costs and makes the process economical and eco-friendly.

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