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Screening of nutritional components for alkaline protease production in submerged fermentation by *Bacillus subtilis* DKMNR using Plackett-Burman design

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ABSTRACT

Screening of fifteen nutrients belonging to two categories,viz., carbon and nitrogen sources was carried out using Plackett-Burman design for the production of alkaline protease by *Bacillus subtilis* DKMNR under submerged conditions. This design involves screening of up to 'n-1' variables in just 'n' number of experiments. Effects, p &t-values were calculated by subjecting the experimental data to statistical analysis. Among selected fifteen nutrients maltose, sucrose, fructose, glucose, peptone, casein, urea and ammonium nitrate showed higher effects and lower p-values. Based on the results in nitrogen sources peptone and in carbon sources glucose were identified as most critical compounds for enhanced alkaline protease production by *B. subtilis* DKMNR.

Keywords: Alkaline protease, Plackett-Burman design (PBD), *Bacillus subtilis* DKMNR, submerged fermentation, carbon and nitrogen, ANOVA.

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INTRODUCTION

Proteases possess some characteristics of biotechnological interest due to which these have become the most important industrial enzymes [5].The demand for alkaline proteases increased due to their application in leather, tannery and many industries. Proteases are produced by both bacteria and fungi among the bacteria *B. subtilis, B. staerothermophilas, B. amyloliquefaciens, B. licheniformis, B. acidocaldarius, Bifidobacteriumbifidum* [1-3] and *B. macerans* [4] were identified as potent protease producers. Increase of protease production by using different nutritional factors such as carbon, nitrogen and other nutrients are of most critical for protease production in an efficient and economic process. The methodologies used for screening the nutrients fall into two major categories; classical and statistical [6]. The statistical methodologies are preferred because of several advantages including in short listing nutrients in rapid and reliable understanding the interactions among the nutrients at varying concentrations, and reduction in number of experiments resulting in saving of time, glassware, chemicals and manpower [7, 8].

Plackett-Burman design (PBD) is a statistical methodology that is used for screening purposes. PBD [9,10] is used to investigate 'n−1' variables in 'n' experiments proposing experimental designs for more than seven factors, and especially for n×4 experiments, i.e. 8, 12, 16, 20 - - - 4n that are suitable for studying up to 7, 11, 15, 19 - - - - 4n-1 factors respectively where 'n' is a multiple of 4 [11]. One useful characteristic is that the sample size is a multiple of 4 rather than a power of 2. There are no two-level fractional factorial designs with sample sizes between 16 and 32 runs. However, there are 20-run, 24-run, and 28-run PBDs. In some cases, where N×4 = 2k, the PBD is a specific fraction of a full factorial design, and saturated fractional factorial designs can be used as well. However, this is not the case for multiples of 4 that are not equal to the power of 2. The main effects are orthogonal and two-factor interactions that are only partially confounded with main effects. This is different from the resolution three-fractional factorial, where two-factor interactions are indistinguishable from main effects. In the PBDs it is also possible to verify that each factor is examined at '+'and '– ' levels.

Comparing further PBD with the fractional factorial designs (FFD), it should be noted that PBD are used when there are more than seven factors, while FFD could be used in situations with less factors. Using the FFD design [12] with Resolution IV, we would need to perform 64 runs, almost five times more experiments, with the gain of the effects of some two way interactions, not necessary in the present case of study. If we wanted to find a modeling equation for predicting the performance of protease then we had to isolate the significant factors from the PBD and then transfer these factors on a FFD to examine the modeling procedure. Furthermore, it should be noted that in PBD one should use dummy factors [13,14] , something not recommended in FFD.

Inspite of the above advantages, the statistical designs are applied to a limited number of submerged fermentation processes for the production of the protease. In the present study,

we report the screening of nutrients using Plackett-Burman design for the production of protease by *B. subtilis* DKMNR under submerged conditions.

MATERIALS AND METHODS

Microorganism and culture condition

The *Bacillus subtilis* DKMNR culture used in this study was isolated from garden soil samples around the department of Chemical Engineering, Andhra University, Visakhapatnam, A.P, India. The production media was composed of yeast extract-peptone-dextrose (YPD) medium consisting ($g.l^{-1}$) of glucose 10.0; peptone, 7.5; yeast extract, 7.5; K_2HPO_4, 0.50, $MgSo_4$ 0.05 and $CaCl_2$ 0.02 and pH 9 after inoculation with 2% (v/v) culture media, incubated at 33^oC on rotary shaker at 200 rpm. The fermentation was carried out for 48 hrs, the culture media was separated by centrifugation and the supernatant was used for assaying enzyme activity.

Estimation of protease Activity

The protease was assayed according to the method of modified Auson-Hagihara method [15]. One unit of alkaline protease activity was defined as 1 µg of tyrosine liberated ml $^{-1}$ under the assay conditions.

Plackett–Burman Design

TABLE 1: SELECTED VARIABLES FOR PB DESIGN

S. No	Compound		levels		
	Real	Coded	-1 (Low)	0	1(High)
1	Starch	X1	0.4	0.2	0.1
2	Maltose	X2	0.4	0.2	0.1
3	Sucrose	X3	0.4	0.2	0.1
4	Glucose	X4	0.4	0.2	0.1
5	Fructose	X5	0.4	0.2	0.1
6	Xylose	X6	0.4	0.2	0.1
7	Galactose	X7	0.4	0.2	0.1
8	Peptone	X8	0.5	0.2	0.1
9	Casein	X9	0.5	0.2	0.1
10	Yeast extract	X10	0.5	0.2	0.1
11	Beef extract	X11	0.5	0.2	0.1
12	Malt extract	X12	0.5	0.2	0.1
13	Urea	X13	0.5	0.2	0.1
14	Amm. Sulphate	X14	0.5	0.2	0.1
15	Amm.nitrate	X15	0.5	0.2	0.1

In order to select the carbon and nitrogen sources for enhancement of the protease production was carried out by employing the PB design. Table 1 indicates the selected variables and their levels. The experimental plan was shown in the table 2. Analysis of the experimental

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results was performed based on the effect of each variable. The effect of each selected variable on protease production was determined using the following equation.

$$E(x_i) = \frac{2\left(\sum Y_i^+ - Y_i^-\right)}{N} \quad ---(1)$$

Where; E (x_i) = the concentration effect of the tested variable.
Y_i^+ and Y_i^- = the protease production from the trials where the variable (x_i) was measured at high and low concentrations, respectively; and N = the number of trials.

The contribution of an ingredient towards the growth of the organism or yield of the enzyme is determined based on the t-value (main effect) calculated from the experimental result [16]. The nutrients are ranked based on their t-values. The nutrient with highest t- value is considered to be the best and ranked one [17] .The sign of the effect indicates the level at which it is considered for further improvement. For example, if a variable have the negative sign means the compound gives the best yield at the low level and experiments should be carried out using further decreased concentration of the compound. All experiments were carried out in triplicate and the average of protease productivity was taken as response (Y). The variables whose confidence levels were higher than 90% were considered to significantly influence on enzyme production.

RESULTS AND DISCUSSION

TABLE 2: PLACKETT-BURMAN EXPERIMENTAL DESIGN ALONG WITH OBSERVED AND PREDICTED PROTEASE YIELD

S. No	X1	X2	X3	X4	X5	X6	X7	X8	X9	X10	X11	X12	X13	X14	X15	Protease Activity (U/mL)
1	-1	-1	-1	-1	1	1	1	1	1	1	-1	-1	-1	-1	1	248
2	1	-1	-1	-1	-1	-1	-1	1	1	1	1	1	1	-1	-1	387
3	-1	1	-1	-1	-1	1	1	-1	-1	1	1	1	-1	1	-1	646
4	1	1	-1	-1	1	-1	-1	-1	-1	1	-1	-1	1	1	1	247
5	-1	-1	1	-1	1	-1	1	-1	1	-1	1	-1	1	1	-1	947
6	1	-1	1	-1	-1	1	-1	-1	1	-1	-1	1	-1	1	1	1055
7	-1	1	1	-1	-1	-1	1	1	-1	-1	-1	1	1	-1	1	267
8	1	1	1	-1	1	1	-1	1	-1	-1	1	-1	-1	-1	-1	450
9	-1	-1	-1	1	1	1	-1	1	-1	-1	-1	1	1	1	-1	555
10	1	-1	-1	1	-1	-1	1	1	-1	-1	1	-1	-1	1	1	610
11	-1	1	-1	1	-1	1	-1	-1	1	-1	1	-1	1	-1	1	850
12	1	1	-1	1	1	-1	1	-1	1	-1	-1	1	-1	-1	-1	954
13	-1	-1	1	1	1	-1	-1	-1	-1	1	1	1	-1	-1	1	1104
14	1	-1	1	1	-1	1	1	-1	-1	1	-1	-1	1	-1	-1	1046
15	-1	1	1	1	-1	-1	-1	1	1	1	-1	-1	-1	1	-1	971
16	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	557
17	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	754
18	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	697
19	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	734
20	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	764

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In the present investigation, the significance of 15 different carbon and nitrogen compounds on production of protease was screened in order to improve the composition of the medium by simultaneous comparisons between two levels (high and low values) of above selected factors by applying the 20 experimental Plackett–Burman design. Table 2 gives the experimental plan along with the results. It was observed that the enzyme production was varied between 247 - 1104 U/mL. It indicates that the selected nutritional compounds show a significant effect on production of the protease.

Based on experimental data, the Pareto chart effects was plotted for identifying the factors that are important in enzyme production in this bacterial strain. This chart show the factors main effect estimates on the horizontal axis. The selected factors main effects are rank ordered according to their significance. The chart also show a vertical line to indicate the statistical significance (P=0.05). If selected variable is significant in the process, the variable-bar crosses the vertical line or vice versa.

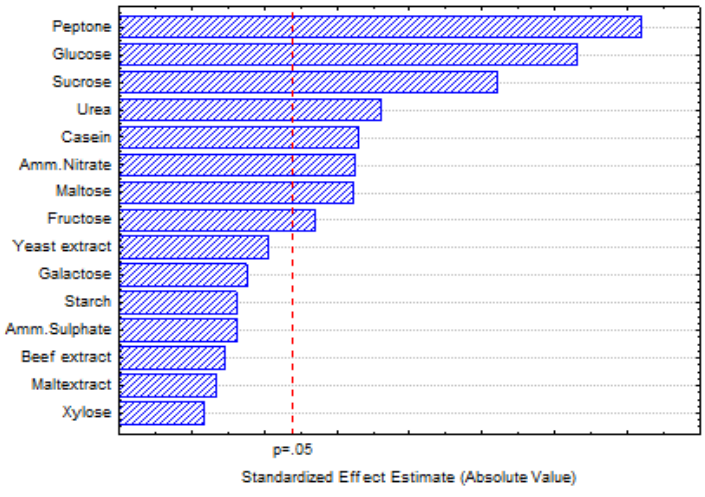

Fig 1: Pareto chart showing the effect of medium components on protease yield

It is evident from the Pareto chart (Fig 1) that more than half of the considered parameters were significantly acting on the system. The carbon sources (glucose, sucrose, maltose and fructose) and nitrogen sources (peptone, urea, casein and ammonium nitrate) are significant, remaining other compounds such as yeast extract, galactose, starch, ammonium sulphate, beef extract, malt extract and xylose were insignificant. Table 3 indicates the ANOVA data from this table it is observed that the peptone has the highest effect (-350.5) and followed by the glucose (300), sucrose (237.5), urea (-147.75), casein (130.5), ammonium nitrate (-127.25), maltose (-126.25) and fructose (-96.25).

The observed lowest p-value (0.00024) and highest F-value (153.601) (F > P value) for peptone indeed suggested that this is the most important nutritional source for the protease production. After the peptone, glucose shows the lowest p-value (0.000447) and highest F-value (112.528). From the table 3, it was observed that peptone, urea, ammonium nitrate, maltose and fructose were showing the highest effect at their lowest concentrations indicates

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that these compounds are required in small amounts for the production of higher titers of protease. It is also evident from the table 2 where the highest activity was observed in the 6th, 13th and 14th runs where peptone was found in the low concentrations. Where as in 1st and 7th runs peptone was found in the higher concentrations at this run the lowest activity was observed.

TABLE 3: EFFECTS AND ANOVA FOR SELECTED VARIABLES

S. No		Effect	Coefficients	SS	df	MS	F	t-value	p-value
1	Mean/ Intercept	692.150	692.150					54.7261	0.000001
2	X1	-35.250	-17.625	4970	1	4970.3	1.5536	-1.2464	0.280616
3	X2	**-126.250**	**-63.125**	**63756**	**1**	**63756.3**	**19.9288**	**-4.4642**	**0.011125**
4	X3	**237.500**	**118.750**	**225625**	**1**	**225625.0**	**70.5254**	**8.3979**	**0.001100**
5	X4	**300.000**	**150.000**	**360000**	**1**	**360000.0**	**112.5281**	**10.6079**	**0.000447**
6	X5	**-96.250**	**-48.125**	**37056**	**1**	**37056.3**	**11.5830**	**-3.4034**	**0.027193**
7	X6	-10.000	-5.000	400	1	400.0	0.1250	-0.3536	0.741490
8	X7	-43.000	-21.500	7396	1	7396.0	2.3118	-1.5205	0.203031
10	X8	**-350.500**	**-175.250**	**491401**	**1**	**491401.0**	**153.6012**	**-12.3936**	**0.000244**
11	X9	**130.500**	**65.250**	**68121**	**1**	**68121.0**	**21.2931**	**4.6144**	**0.009922**
12	X10	-60.250	-30.125	14520	1	14520.3	4.5387	-2.1304	0.100160
13	X11	26.000	13.000	2704	1	2704.0	0.8452	0.9194	0.409934
14	X12	19.500	9.750	1521	1	1521.0	0.4754	0.6895	0.528415
15	X13	**-147.750**	**-73.875**	**87320**	**1**	**87320.3**	**27.2944**	**-5.2244**	**0.006408**
16	X14	35.250	17.625	4970	1	4970.3	1.5536	1.2464	0.280616
17	X15	**-127.250**	**-63.625**	**64770**	**1**	**64770.3**	**20.2458**	**-4.4995**	**0.010826**
18	Error			12797	4	3199.2			
19	Total SS			1447329	19				

The probability plot of effects is very useful for separating random noise from 'real' effects based on their distribution on the plot which is constructed as follows; first the effect estimates are rank ordered. From these ranks, z values (i.e. standard values of the normal distribution) can be computed based on the assumption that the estimates come from a normal distribution with a common mean. These z values are plotted on the left y-axis in the plot and the corresponding normal probabilities are shown on the right y-axis in the plot. The effects are plotted on the x- axis. It is assumed that true effect parameters are separated as outliers.

It is evident from (Fig 2) that among selected variables peptone and glucose were seen as outliers with positive mean values separated from other variables. The outlier's variables have the more positive influence on the protease production. This suggested that further optimization of these variables improves the enzyme production in this *B. subtilis* DKMNR. Based on the above inference for further studies the peptone and glucose concentrations were selected to optimize the higher production of protease.

Nitrogen source plays a major role on protease secretion. Peptone and yeast extract enhances the protease production by *Bacillus* sp [18]. Similarly yeast extract and peptone combination enhanced the enzyme production in the fungus. However the role of urea on

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production of protease by *B. Subtilis* DKMNR differs from the Adidi et al [19] and Chellappan et al [20]. The authors observed the inhibition of protease with addition of urea.

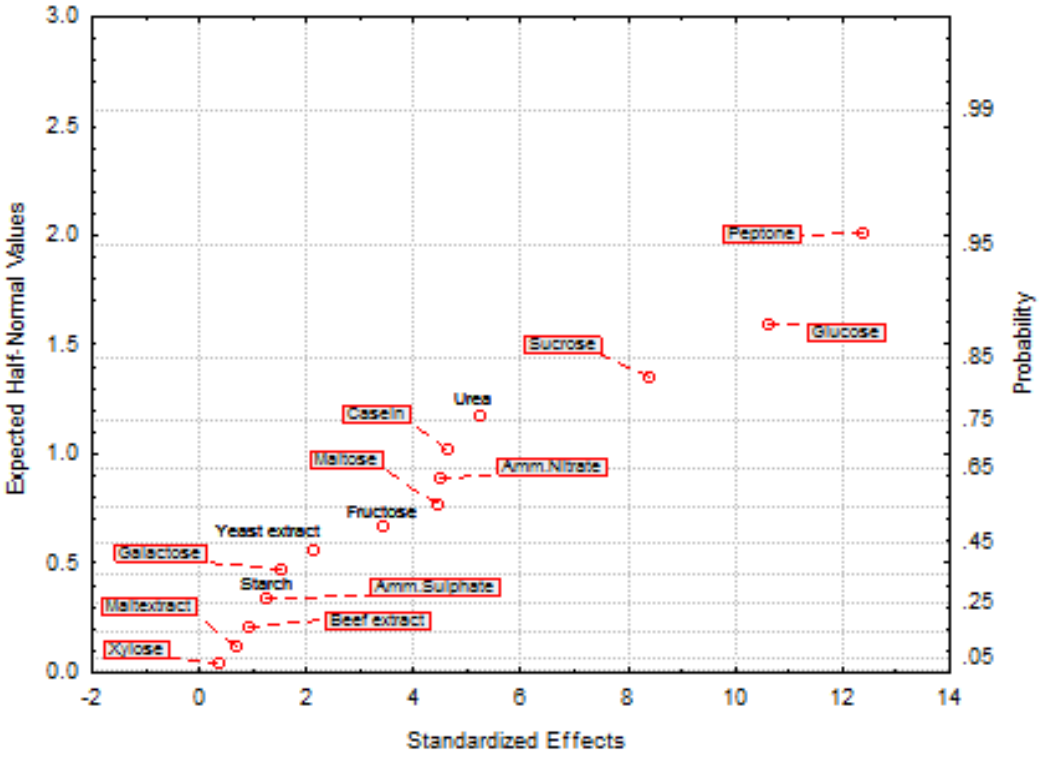

Fig 2: The probability plot of effects on protease production.

In the literature the researchers has a different experiences with the role of glucose on protease production. It was observed that it has a catabolic repression on the protease production. Joo et al, [23] Joo and Chang [24] reported the catabolic repression of the glucose on the protease production in *Botrytis cinerea* and *Bacillus sp.* respectively. Where as Subbarao et al [18, 21, 22] reported that glucose mediated enhanced enzyme production .The complex sugars such as lactose has increased the protease production in *Bacillus sp* [25]. In the present study *B. subtilis* DKMNR has not utilized the complex sugars such as galactose. The PBD results analysis shows that galactose was not a significant compound.

The *B. subtilis* DKMNR differs with other *Bacillus sp* in the utilization of the starch. The PBD results indicated that starch doesn't have any significant effect on protease production. Where in the literature Adidi et al [19] and Chang [24] observed the enhanced protease production with fungus and bacteria. Mehta et al [26] and Darmwal et al [27] used molasses as an alternative to glucose and they observed the enhanced protease production. Whereas Damare et al [28] found to be molasses was repressed the protease production by fungus. From the literature reports and present study we could understand that the utilization of the carbon sources for enhanced protease production is organism specific rather than the substratum. Hymavathi et al [29] used the PBD for screening of the carbon and nitrogen sources for L-asparaginase production.

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CONCLUSION

In this investigation, Plackett-Burman design offers a good and fast screening procedure to test the relative importance of medium components on the production of protease. Among the variables glucose and peptone were found to be the most important variables. The optimum levels of the variables can be determined using response surface methodology in further research.

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