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Plackett-Burman design for screening media components for alkaline protease production from *Streptomyces pulveraceus* through solid state fermentation

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ABSTRACT

Plackett-Burman design for alkaline protease production through solid state fermentation was investigated in this study. The various parameters which were screened for in this study are Temperature, moisture content, pH, I.C, NaCl and various carbon and nitrogen sources. Those are temperature, moisture content, pH, Inoculum concentration, Nacl concentration, Maltose concentration and yeast extract. Further analysis of the Pareto chart denoted that none of the selected variables showed the statistical significance on enzyme production. The least significant factors among selected variables were fructose and tryptone. Lowest p-value (0.000199) for temperature indeed suggested that this is the most important parameter for the protease production. For further studies the temperature, inoculum concentration, moisture content, pH, NaCl, maltose and yeast extract concentrations were selected to optimize the production of production.

Key words: Plackett Burmann design, protease, Temperature, pH, carbon and nitrogen sources

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INTRODUCTION

Actinomycetes are gram positive, filamentous bacteria with a fungal type of morphology and assumed to be the transition group between fungi and bacteria. The features in which they resemble bacteria include diameter of hyphae about 1 μm, prokaryotic nature, sensitivity to phages, lysine synthesis through the diaminopimelic acid pathway, lack of sterols, mucopeptides in cell walls sensitivity to antibacterial antibiotics and immunological relationships. The fungal characteristics they share include hyphae with true branching, formation of mycelium or pellets when grown in broth and production of spores for dissemination. Most of the actinomycetes can be found with greater or less frequency in most ecological settings. They occur in high numbers in the top few inches of the soil and decrease with depth. Actinomycetes play an important role in the decomposition of organic matter in soil. They are the producers of most of the bioactive metabolites and industrially important enzymes. They include numerous potentially useful compounds providing the widest range and most promising array of pharmacologically and agriculturally active compounds. The quantitative ability to secrete proteolytic enzymes could be measured by the degrees of gelatin liquefaction or casein hydrolysis. The proteolytic enzymes produced from *Streptomyces* are more thermotolerant than corresponding animal enzymes. An active protease enzyme with potency equal to that of pancreatin was obtained in a medium containing two percent of soy bean meal produced by *S. griseus*. *Streptomycetes* proteases have been used for the structural determination of other proteins and also for removing proteinaceous material during purification of certain bio preparations. For commercial purposes they are routine

obtained as byproducts formed during biosynthesis of antibiotics [8] mostly from fermentation broth of *Streptomyces fradiae*, *S.griseus* and *S.rimosus* [10].

These enzymes are mostly active at pH-8 and have a molecular weight of around 20kD. Proteases isolated from *S. rectus* [5] and *S. diastaticus* strain SS1 were identified as a thermo stable alkaline metalloproteases [1] . Proteolytic enzymes produced from Streptomyces sps were reported to effectively dehair hides and skin. The *Streptomyces* strains studied that showed varying degree of proteolytic activities were *S.griseus(5 strains), S. griseoflavus (2 strains) S. cellulosae (5 strains), S.fulvassimus(3 strain) S.olieaceous (5 strains) S. diastatochromogenus(3 strains) S.bobiliae (2strains), S.saureus (2strains) S. pheochromogenus and S.erythrochrmogemus.* Solid State fermentation process can be defined as the growth of microorganisms on moist solid material which has been applied for production of enzymes and organic acids using mold culture [7]. In SSF certain solid substrates not only supply nutrients to the culture but also serve as an anchorage to microbial cells. Positive influence is shown when the supports are supplemented with other ingredients containing growth factors and salts. Substrates that have been traditionally used in solid state fermentation include a variety of agriculture products such as rice, wheat, barley millet, barley grains, beans, corn and soyabean.

Traditionally SSF was carried out through batch fermentation using natural heterogeneous material. SSF process is operated in batch, fed batch or continuous modes, but batch processes are most common. Critical analysis of literature reveals a number of reasons for resurgence of interest in SSF. Presently SSF is applied in large scale processes mainly in Thailand and Japan. It is estimated that nearly one third of industrial SSF and Koji processes in Japan has been modernized for large scale production of citric acid and itaconic acid using wheat bran as solid substrate. It is evident from above that alkaline proteases have a wide range of industrial applications. In addition to the applications already described, alkaline proteases are also used to lesser extent in a large number of other fields, which may be technically interesting, but are not commercial success in terms of microbial enzyme sales: nevertheless they are the upcoming areas of future enzyme industry. Thangam and Rajkumar (2002) [9] reported that some proteases can replace Proteinase K. Chiplonkar *et. al.* (1985)[2] have reported the use of alkaline protease from *Conidiobolus* sp. as a substitute for trypsin in preparation of animal cell cultures. In another study, the effect of culture supernatant of *M. purpureus* CCRC 31499 on the growth rate of rape and amaranth seedlings was investigated [4]. PB design of tannase production was investigated by Mohan *et al*.(2013) [6] . Since, medium optimization by single dimensional search is time consuming, hence PB method was used in screening experiment [3].

EXPERIMENTAL SECTION

Sample collection:
Samples were collected from various places located in and around Warangal, AP, India. Soil samples were collected from dump yards of Food Corporation of India, dairy farms and drainage of slaughterhouse.

Isolation and screening of organisms:
One gram of soil sample was suspended in 9ml of sterile distilled water, and 1 ml of suspension was used to inoculate 50 ml of screening medium (starch, 10 g/L; peptone, 5 g/L; yeast extract, 5 g/L; KH2PO4, 10 g/L; NaCl 5.0 g/L) pH of the medium was adjusted to 9.0. The culture was incubated at 30°C for 48 hrs at 150 rpm. The culture was serially diluted and 0.1 ml of culture was plated on screening medium for isolation of pure cultures.

Screening for proteolytic activity:
Proteolytic activity from isolated pure cultures was screened by plating on Casein agar [Casein 0.3g, KNO_3 0.2g, NaCl 0.2g, K_2HPO_4 0.2g, $MgSO_4$ 0.005g, $CaCl_2$ 0.002g, $FeSO_4$ 0.001g, Yeast extract, 0.1g; agar 2g, distilled water 100ml]. Plates were incubated at 30°C for 24 hrs. Bacteria producing clear zones of caesinolysis on casein agar plates were identified as protease producers. The ratio of hydrolysis zone/growth zone was calculated which gives a measure of the caesinolytic activity. The strain producing maximum caesinolytic activity was selected and maintained on nutrient agar slants/glycerol cultures at 4°C.

Characterization of protease producing isolate:
The cultural, morphological and physiological characteristics of isolate S-1 were investigated by using various media and biochemical reactions. The isolate S-1 was compared and identified according to Bergey's Manual of Determinative Bacteriology and confirmed by MTCC Chandigarh. To select the process as well as nutritional

parameters a PB designs was employed. Table 1 indicates the selected variables and their levels. The experimental plan was shown in the table 2. Analysis of the experimental results was performed based on the effect of each variable. The effect of the each selected variable on protease production was determined using the following equation.

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

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

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 responses (Y). The variables whose confidence levels were higher than 90% were considered to significantly influence on protease production.

RESULTS AND DISCUSSION

In the present investigation, the significance of 15 different process and nutritional parameters 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 Plackett–Burman design to the 16 experimental parameters. Table 3 shows the experimental setup with the results. The Pareto chart of effects was plotted for identifying the factors that are important in enzyme production in this *Streptomyces pulveraceus* based on experimental data. This chart show the factors main effect estimates on the horizontal axis. The statistical significance (P=0.05) is shown as a vertical line in chart. If selected variable is significant , the variable-bar crosses the vertical line. Pareto chart of carbon (fig 1) identified the most important parameters were identified which are Temperature, moisture content, pH, Inoculum concentration, NaCl concentration, Maltose concentration and yeast extract. From the Pareto chart (Fig 1) none of the selected variables showed the statistical significance on enzyme production. ANOVA was used for further confirmation. From Table 3, the F statistics and P values are not available indicated that none of the selected variables have the impact on protease production. To obtain the standardized effects, F and P values from saturated PBD, the most insignificant two factors from each group based on Pareto chart were ignored. The least significant factors of among selected variables, fructose and tryptone were selected for pooling their effects into error. The transformed ANOVA data is shown in Table 4.

Table 1 : Different variables and their levels

S. No	Variable		'-' level	'+' level
	Real	Coded		
1	Temperature	Te	33	37
2	Moisture content	Mo	100	120
3	pH	pH	8	9
4	I.C	I.C	1.5	2.5
5	Nacl	Na	0.5	0.75
6	Maltose	Ma	1	1.5
7	Fructose	F	0.2	0.5
8	Sucrose	Su	0.2	0.5
9	Starch	St	0.2	0.5
10	Arabinose	Ar	0.2	0.5
11	Xylose	Xy	0.2	0.5
12	Yeast extract	Y.E	0.1	0.2
13	Peptone	Pe	0.1	0.2
14	Beef extract	B.E	0.1	0.2
15	Tryptone	Tr	0.1	0.2

Table 2: The Plackett–Burman experimental design matrix for screening of process and nutritional parameters for protease production

. No	Te	Mo	pH	I.C	Na	Ma	F	Su	St	Ar	Xy	Y.E	Pe	B.E	Tr
1	-1	-1	-1	-1	1	-1	1	1	-1	1	1	1	-1	1	-1
2	-1	-1	1	1	-1	1	-1	1	-1	1	-1	1	1	-1	1
3	1	-1	1	1	-1	-1	1	1	-1	-1	1	-1	-1	-1	1
4	1	-1	-1	1	-1	-1	1	-1	1	1	-1	-1	1	1	-1
5	1	-1	-1	-1	1	1	-1	-1	1	1	1	-1	-1	-1	1
6	-1	1	-1	1	1	-1	-1	1	1	-1	-1	-1	-1	1	1
7	-1	1	-1	-1	-1	1	1	1	1	-1	1	-1	1	-1	-1
8	-1	1	1	-1	-1	1	1	-1	-1	1	-1	-1	-1	1	1
9	1	1	-1	1	1	1	1	-1	-1	-1	-1	1	-1	-1	-1
10	1	1	1	-1	-1	-1	1	1	1	1	-1	1	-1	-1	-1
11	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
12	1	-1	1	-1	1	1	-1	1	-1	-1	-1	-1	1	1	-1
13	1	1	-1	-1	-1	-1	-1	-1	-1	-1	1	1	1	1	1
14	-1	-1	1	1	-1	1	-1	-1	1	-1	1	1	-1	1	-1
15	-1	1	1	1	1	-1	-1	-1	-1	1	1	-1	1	-1	-1
16	-1	-1	1	-1	1	-1	1	-1	1	-1	-1	1	1	-1	1

Table 3: The Plackett–Burman experimental design matrix along with the protease activity

S. No	Te	Mo	pH	I.C	Na	Ma	F	Su	St	Ar	Xy	Y.E	Pe	B.E	Tr	Protease activity		
																Observed	Predicted	Error
1	33	120	6	1.5	0.75	1	0.5	0.5	0.2	0.5	0.5	0.2	0.1	0.2	0.1	27003.75	26898.17	105.585
2	33	120	7	2.5	0.5	1.5	0.2	0.5	0.2	0.5	0.2	0.2	0.2	0.1	0.2	27571.65	27712.43	-140.77
3	37	120	7	2.5	0.5	1	0.5	0.5	0.2	0.2	0.5	0.1	0.1	0.1	0.2	18438.30	18366.83	71.466
4	37	120	6	2.5	0.5	1	0.5	0.2	0.5	0.5	0.2	0.1	0.2	0.2	0.1	15025.65	14920.07	105.585
5	37	120	6	1.5	0.75	1.5	0.2	0.2	0.5	0.5	0.5	0.1	0.1	0.1	0.2	12887.40	13028.18	-140.77
6	33	140	6	2.5	0.75	1	0.2	0.5	0.5	0.2	0.2	0.1	0.1	0.2	0.2	19326.75	19467.53	-140.77
7	33	140	6	1.5	0.5	1.5	0.5	0.5	0.5	0.2	0.5	0.1	0.2	0.1	0.1	10597.20	10491.62	105.585
8	33	140	7	1.5	0.5	1.5	0.5	0.2	0.2	0.5	0.2	0.1	0.1	0.2	0.2	13447.65	13376.18	71.466
9	37	140	6	2.5	0.75	1.5	0.5	0.2	0.2	0.2	0.2	0.2	0.1	0.1	0.1	10012.95	9907.37	105.585
10	37	140	7	1.5	0.5	1	0.2	0.5	0.5	0.5	0.2	0.2	0.1	0.1	0.1	9844.50	9880.77	-36.271
11	37	140	7	2.5	0.75	1.5	0.5	0.5	0.5	0.5	0.5	0.2	0.2	0.2	0.2	19935.75	19864.28	71.466
12	37	120	7	1.5	0.75	1.5	0.2	0.5	0.2	0.2	0.2	0.1	0.2	0.2	0.1	13039.35	13075.62	-36.271
13	37	140	6	1.5	0.5	1	0.2	0.2	0.2	0.2	0.5	0.2	0.2	0.2	0.2	7117.95	7258.73	-140.77
14	33	120	7	2.5	0.5	1.5	0.2	0.2	0.5	0.2	0.5	0.2	0.1	0.2	0.1	29052.30	29088.57	-36.271
15	33	140	7	2.5	0.75	1	0.2	0.2	0.2	0.5	0.5	0.1	0.2	0.1	0.1	31134.60	31170.87	-36.271
16	33	120	7	1.5	0.75	1	0.5	0.2	0.5	0.2	0.2	0.2	0.2	0.1	0.2	31680.00	31608.53	71.466

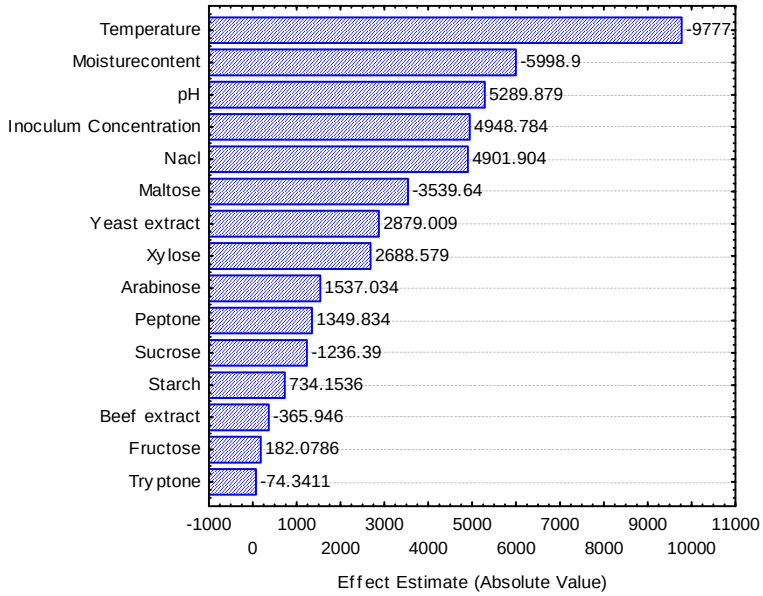
Table 4: Effects of various parameters using ANOVA

Parameter	Effect	SS	df	MS	F-Value	p-Value
Mean/Interc.	18176.62	-	-	-	-	-
Temperature	-9777.77	374770871	1	374770871	-	-
Moisture content	-5998.90	141068093	1	141068093	-	-
pH	5289.88	85697371.9	1	85697372	-	-
Inoculum Concentration	4948.78	96002612.5	1	96002612	-	-
Nacl	4901.90	94192341.8	1	94192342	-	-
Maltose	-3539.64	49113910.9	1	49113911	-	-
Fructose	182.08	129958.216	1	129958	-	-
Sucrose	-1236.39	5992358.5	1	5992358	-	-
Starch	734.15	2112807.35	1	2112807	-	-
Arabinose	1537.03	9260895.33	1	9260895	-	-
Xylose	2688.58	28335542.6	1	28335543	-	-
Yeast extract	2879.01	32491674.3	1	32491674	-	-
Peptone	1349.83	7142442.41	1	7142442	-	-
Beef extract	-365.95	524953.811	1	524954	-	-
Tryptone	-74.34	21664.252	1	21664	-	-
Error		0	0			
Total SS		1039923347	15			

Table 5: Transformed ANOVA for the effect of various parameters

Parameters	Effect	SS	df	MS	F	p
Mean/Interc.	18179.13					
Temperature	-9782.80	375450864	1	375450864	5020.744	0.000199
Moisture content	-6003.92	141415572	1	141415572	1891.090	0.000528
pH	5249.66	87843951.9	1	87843952	1174.700	0.000850
Inoculum Concentration	4953.81	96273292.4	1	96273292	1287.422	0.000776
NaCl	4896.88	94072999.3	1	94072999	1257.998	0.000794
Maltose	-3534.61	49012925.1	1	49012925	655.429	0.001522
Sucrose	-1231.36	5948386.64	1	5948387	79.545	0.012339
Starch	729.13	2085603.17	1	2085603	27.890	0.034035
Arabinose	1542.06	9328898.37	1	9328898	124.751	0.007921
Xylose	2683.55	28251820.9	1	28251821	377.800	0.002636
Yeast extract	2884.04	32630851.6	1	32630852	436.358	0.002284
Peptone	1354.86	7201397.73	1	7201398	96.301	0.010225
Beef extract	-370.97	539901.32	1	539901	7.220	0.115084
Error		149559.861	2	74780		
Total SS		1039923347	15			

Fig 1: Pareto chart of effects



Analysis of the transformed ANOVA data (Table 5) and Figure 2 indicated that beef extract is also insignificant for the protease production among selected variables in this bacterial strain. Lowest p-value (0.000199) for temperature indeed suggested that this is the most important parameter for the protease production. The probability plot of effects is very useful for separating random noise from 'real' effects based on their distribution on the plot. The effect estimates are rank ordered and 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 evident from fig 3 that among selected variables, temperature, moisture content and maltose concentration were positioned as outliers with negative mean values. The parameters pH, inoculum and NaCl concentration were seen as outliers with positive mean values separated from other variables. the outliers variables have the more positive influence on the protease production. This suggested that further optimization of these variables improves the protease production in this *Streptomyces pulveraceus* For further studies the temperature, inoculum concentration, moisture content, pH , NaCl, maltose and yeast extract concentrations were selected to optimize the production of production.

Fig 2: Pareto chart of effects after two factors pooled in to error

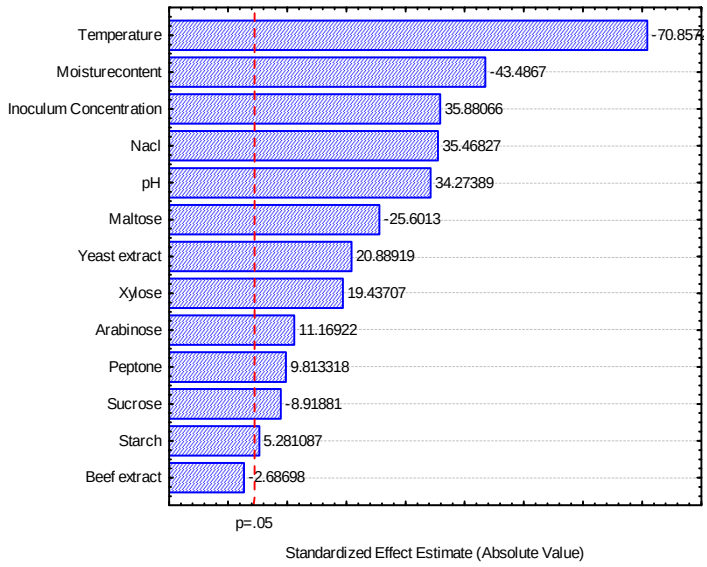
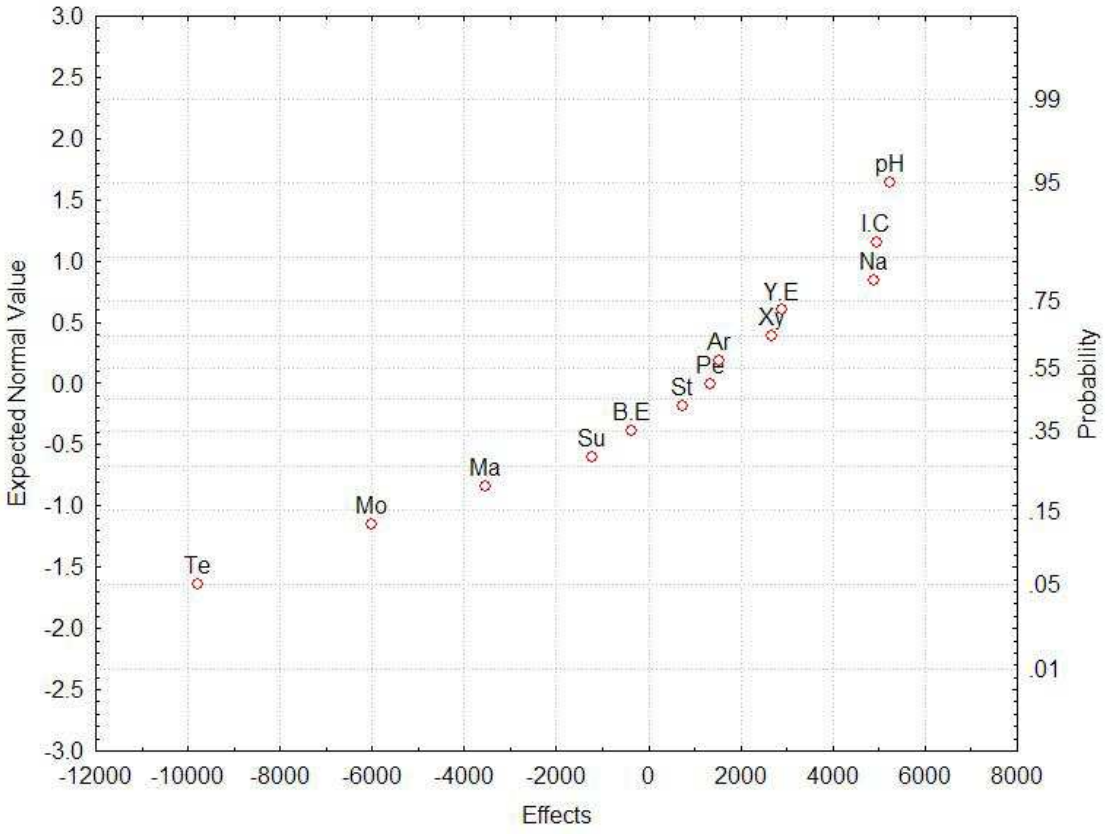


Fig 3: Normal probability plot of selected variables on protease production



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