Screening of medium components for production of L-Glutaminase by isolated *Aspergillus* sp using Plackett–Burman design

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Abstract:

Screening of eleven nutrients belonging to two categories, viz., carbon and nitrogen sources was carried out using Plackett-Burman design for the production of glutaminase by isolated *Aspergillus* sp 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 eleven nutrients $NaNO_3$, lactose and yeast extract showed higher effect and lower p-values. Based on the results in nitrogen sources $NaNO_3$ and in carbon sources lactose were identified as a potential glutaminase enhancing nutrients.

Key words: Glutaminase, Plackett-Burman, Screening, Medium

1. INTRODUCTION:

L-Glutaminase (L-glutamine amidohydrolase EC 3.5.1.2) catalyzes the hydrolysis of L-glutamine to L-glutamic acid and ammonia. This is an essential enzyme for synthesis of various nitrogenous metabolic intermediates. The glutaminase also acts as a catabolic repressor in microorganisms. It is also present in the mammalian tissues and break downs the glutamine into glutamic acid and ammonia . There is enormous scope exists for the search for potential strains that could produce L-glutaminase in high yields and with novel properties under economically viable bioprocesses. Wade et al, Nelson et al and Imada et al compiled more comprehensive lists of glutaminase producing organisms.

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 . 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 .

Plackett-Burman design (PBD) is a statistical methodology that is used for the screening purposes. PBD are 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, etc., that are suitable for studying up to 7, 11, 15, 19, etc. factors, respectively. 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. Let us consider the case of 12 experiments for 11 factors as happens in the present study. It is also possible to verify that each factor is examined at six + and six- 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 of reference 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

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performance of liposomes then we had to isolate the significant factors from the PBD and then to transfer these factors on a FFD to examine the modeling procedure. Furthermore, it should be noted that in PBD one should use dummy factors , something not recommended in FFD.

In spite of the above advantages, the statistical designs are applied to a limited number of submerged fermentation processes, SSF processes and never attempted earlier for the production of the glutaminase. In the present study, we report the screening of nutrients using Plackett-Burman design for the production of glutaminase by isolated *Aspergillus* sp under submerged conditions.

2. MATERIALS AND METHODS:

Micro organism and cultural condition:

The *Aspergillus* culture used in this study was isolated from marine water of the Bay of Bengal along the Visakhapatnam coast. The initial production medium was composed of KH_2PO_4 1 g, $MgSO_4$ 0.5 g, $CaCl_2$ 0.1 g, $NaNO_3$ 0.1 g, Trisodium citrate 0.1 g, NaCl 10.0 g, L-glutamine 10.0 g, Dextrose 5.0 g, Sea water 1lit and pH 6.0 After inoculation with a 2% (v/v) conidial suspension, incubated at 27 °C on a rotary shaker at 120 rpm.Initially the fermentation was carried out for 48 hours, the mycelia was separated by centrifugation and the supernatant was used for assaying enzyme activity.

Estimation of glutaminase activity:

Glutaminase was assayed according to the method of Imada et al.where the liberated ammonia due to the action of enzyme was estimated using Nessler's reagent.

Plackett–Burman Design

In order to select the additional carbon and nitrogen source for enhancement of the glutaminase 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 results was performed based on the effect of each variable. The effect of the each selected variable on glutaminase 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 glutaminase 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 glutaminase productivity was taken as responses (Y). The variables whose confidence levels were higher than 90% were considered to significantly influence on enzyme production.

3.Results and discussion:

In the present investigation, the significance of 11 different carbon and nitrogen compounds on production of glutaminase 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 12 experimental Plackett–Burman design. Table 2 gives the experimental plan along with the results. It was observed that the enzyme production was varied between the 1.37-32 U/ml, it indicates that the selected nutritional compounds show a significant effect on the production of the glutaminase.

The experimental data was further analyzed. It was observed that the design was saturated. From the saturated design the significant factors and other parameters determination is difficult. In order to convert saturated design to unsaturated design the least effect variables were pooled into the error. In the present study the maltose and galactose were found to be least significant factors. In order to get the error variability and convert saturated design to unsaturated design these two factors were pooled into error.

Based on experimental data, the Pareto chart of effects was plotted for identifying the factors that are important in enzyme production in this fungal 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.

It is evident from the Pareto chart (fig 1) that the most important parameters are $NaNO_3$ followed by lactose and yeast extract remaining other compounds are insignificant.

Table 3 indicates the ANOVA data, from this table it is observed that the $NaNO_3$ has the highest effect (-10.1050) and followed by the lactose (-7.1083) and yeast extract (-6.7583).

The observed lowest p-value (0.0176) and highest F-value (55.125) (F > P value) for $NaNO_3$ indeed suggested that this is the most important nutritional source for the glutaminase production. After the sodium nitrate lactose shows the lowest p-value (0.0347) and highest F-value (27.278). Figure 2 depicts the levels plot of the selected variables it was observed that both sodium nitrate and lactose were shown the highest effect at their lowest concentrations indicates that these compounds are required in a small amounts for the production higher concentrations negatively effect the production it is also evident from the table where the highest activity was observed in the 12^{th} and 3^{rd} runs where both lactose and $NaNO_3$ found in the low concentrations. Where as in the 10^{th} run both compounds are in the higher concentrations at this run the lowest activity was observed.

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 3 that among selected variables $NaNO_3$, lactose and yeast extract were positioned as outliers with negative mean values. Meat extract was seen as outliers with positive mean values separated from other variables. The outlier's variables have the more positive influence on the glutaminase production. This suggested that further optimization of these variables improves the enzyme production in this *Aspergillus* sp. Based on the above inference for further studies the $NaNO_3$ and lactose concentrations were selected to optimize the higher production of glutaminase. Sathish et al were used the statistical designs (mixed designs) used for the production of the glutaminase in the solid state fermentation.

Table 1: Selected Variables for PB Design

S. No	Variable	Low (-) (mg)	High (+)(mg)
1	Glucose	100	200
2	Lactose	100	200
3	Maltose	100	200
4	Starch	100	200
5	Sucrose	100	200
6	Galactose	100	200
7	$NaNO_3$	100	200
8	Yeast extract	50	150
9	Casein	50	150
10	Peptone	50	150
11	Meat extract	50	150

Table 2 : PB design along with observed and predicted glutaminase yield.

Run No	Glucose	Lactose	Maltose	Starch	Sucrose	Galactose	$NaNO_3$	yeast extract	casein	peptone	Meat extract	Glutaminase activity (U/ml)		
												Observed	Predicted	Error
1	1	-1	1	-1	-1	-1	1	1	1	-1	1	7.9	8.63	-0.73
2	1	-1	-1	1	-1	1	-1	1	1	1	-1	8.22	7.48	0.74
3	-1	-1	1	-1	1	1	-1	-1	1	1	1	24.67	25.81	-1.14
4	1	1	1	1	-1	-1	-1	-1	-1	1	1	10.96	11.69	-0.73
5	1	-1	-1	1	1	1	1	-1	-1	-1	1	10.51	9.77	0.74
6	1	1	1	-1	1	1	-1	1	-1	-1	-1	7.77	8.91	-1.14
7	-1	1	1	1	-1	1	1	-1	1	-1	-1	5.03	6.17	-1.14
8	-1	-1	1	1	1	-1	1	1	-1	1	-1	2.29	3.02	-0.73
9	-1	1	-1	1	1	-1	-1	1	1	-1	1	10.96	9.81	1.15
10	1	1	-1	-1	1	-1	1	-1	1	1	-1	1.37	0.22	1.15
11	-1	1	-1	-1	-1	1	1	1	-1	1	1	6.85	6.11	0.74
12	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	32.00	30.85	1.15

Table 3: Effects and ANOVA for selected variables.

	Effect	t-value	p-value	F-value	SS	df	MS
Mean/Interc.	10.7108	15.73954	0.004012	-	-	-	-
Glucose	-5.8450	-4.29461	0.050174	18.44364	102.4921	1	102.4921
Lactose	-7.1083	-5.22284	0.034759	27.27804	151.5852	1	151.5852
Starch	-5.4317	-3.99091	0.057430	15.92736	88.5090	1	88.5090
Sucrose	-2.2317	-1.63971	0.242742	2.68866	14.9410	1	14.9410
$NaNO^3$	-10.1050	-7.42464	0.017661	55.12521	306.3331	1	306.3331
Yeast extract	-6.7583	-4.96568	0.038244	24.65794	137.0252	1	137.0252
Casein	-2.0383	-1.49766	0.272927	2.24299	12.4644	1	12.4644
Peptone	-3.3017	-2.42590	0.136083	5.88497	32.7030	1	32.7030
Meat extract	2.5283	1.85769	0.204327	3.45101	19.1774	1	19.1774
Error					11.1141	2	5.5570
Total SS					876.3445	11	

Fig1: Pareto chart of effects of selected compounds on glutaminase yield

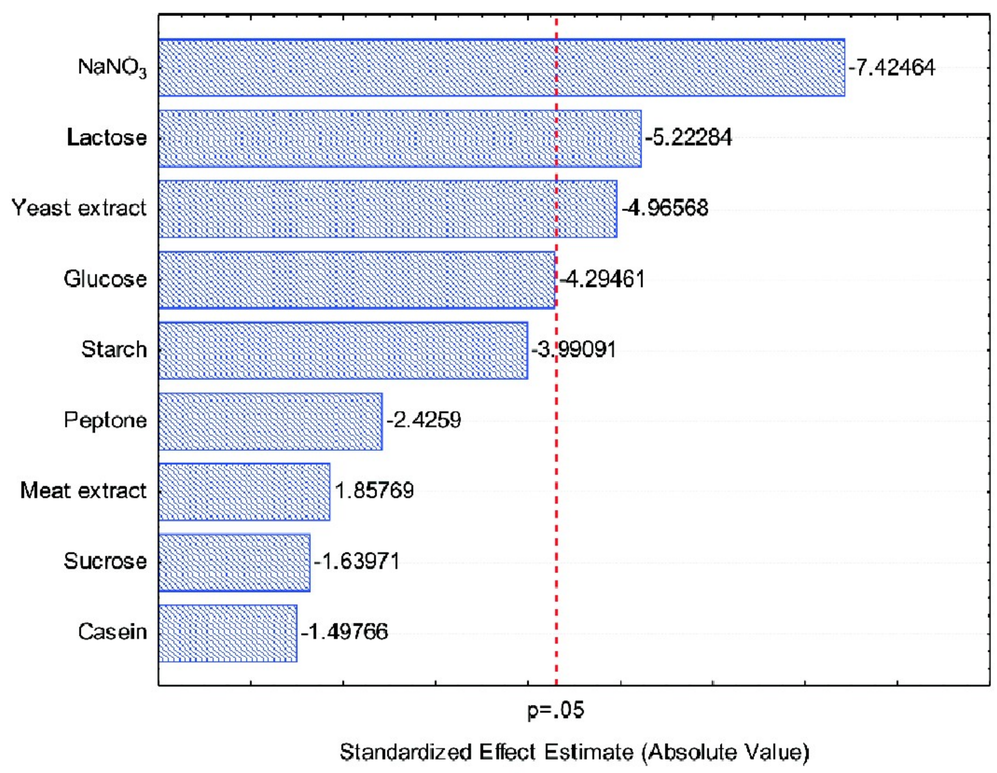


Fig 3: The probability plot of effects for glutaminase production.

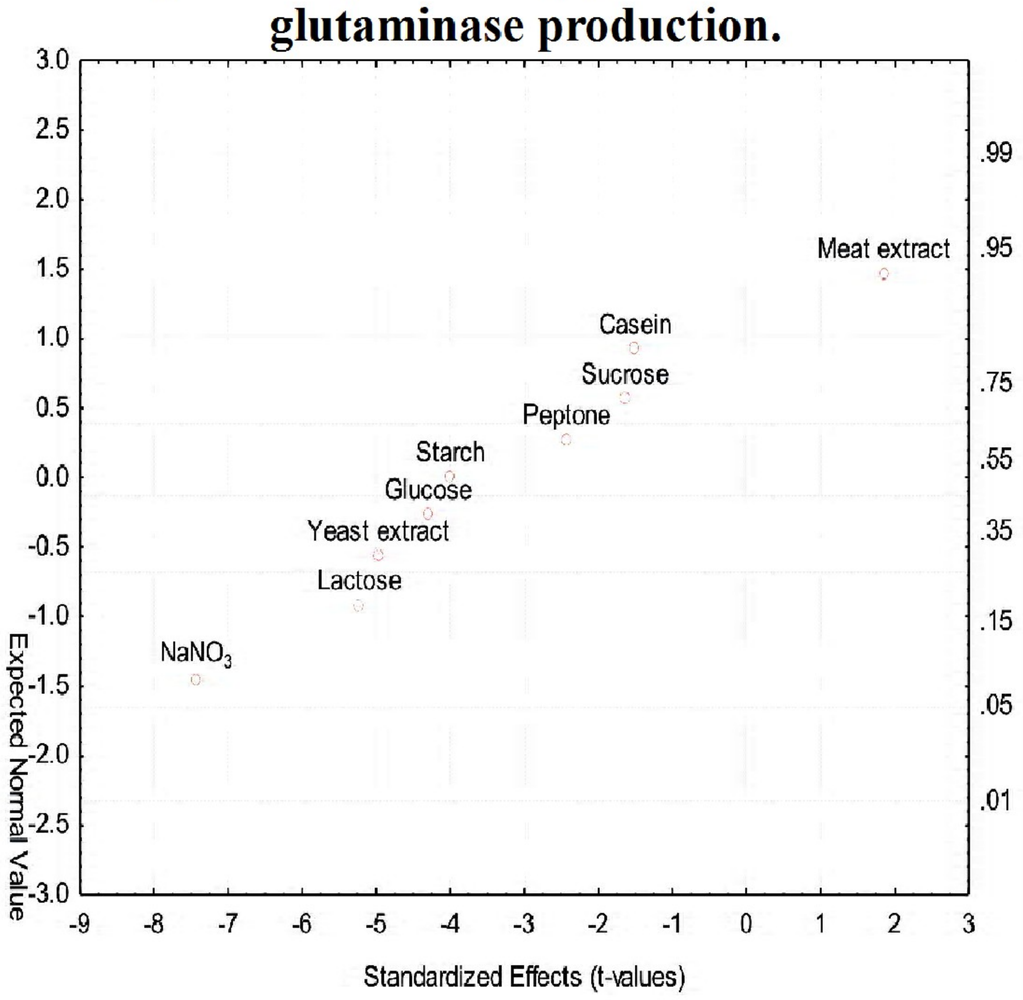
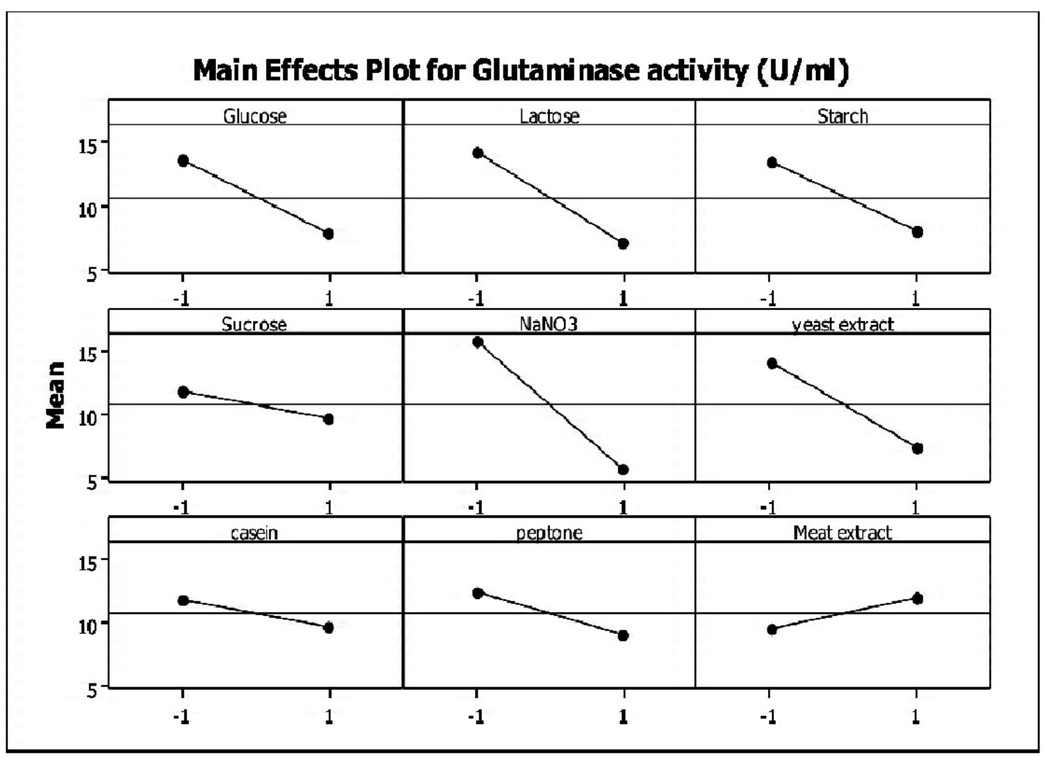


Fig 2: Main effects plot for glutaminase activity



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