# Impact of Carbon and Nitrogen Sources on L-Asparaginase Production by Isolated *Bacillus circulans* (MTCC 8574): Application of Saturated Plackett-Burman Design

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The present work aimed to study the impact of different carbon and nitrogen sources on the effective production of L-asparaginase by isolated *Bacillus circulans* using Plackett-Burman design (PBD).

Among carbon sources; lactose, galactose, starch, sucrose, maltose, arabinose and among nitrogen sources; proline, tryptone, sodium glutamate, corn steep liquor, beef extract, ammonium chloride, yeast extract at selected concentration produced a maximum of 105 and 164 U $mL^{-1}$ L-asparaginase enzyme, respectively. Initial analysis of L-asparaginase data with different carbon and nitrogen sources as per PBD did not reveal standardized effects as well as '*p*' and '*t*' values. Regression coefficient and *t*-values were calculated by subjecting the experimental data to statistical analysis after pooling the least two nutrient components into error. Among selected 26 components, xylose, galactose, yeast extract and proline denoted least significance, while glucose, mannose, ammonium chloride and ammonium sulphate showed maximum significance on enzyme production.

Saturated PBD can be effectively utilized for analysis of the data by pooling the least significant factors based on the effects on metabolite/product/enzyme production. Ammonium chloride and glucose were observed to be the most significant carbon and nitrogen sources, respectively in L-asparaginase production in this bacterial strain. The presented approach is highly useful in bioprocess optimization procedure where all selected parameters show little degree of freedom and subsequent significant factors.

*Key words:*
L-Asparaginase, *Bacillus circulans*, saturated Plackett-Burman design, optimization

## Introduction

L-Asparaginase (L-asparagine amino hydrolase, E.C.3.5.1.1) catalyzes the irreversible hydrolysis of L-asparagine to aspartic acid and ammonium ions. This enzyme has gained importance in the pharmaceutical sector and has been clinically accepted as an anti-tumor agent for the effective treatment of acute lymphoblastic leukemia and lymphosarcoma.[1,2] At present, this enzyme is administered in combination with other chemical drugs for treating certain types of cancers. Hence, the demand for L-asparaginase is increasing daily with the rising disease incidence in the world.

Almost all living cells produce L-asparaginase but only certain microbial strains have the potential for industrial production of this enzyme. Hence, several microbial strains belong to bacteria *Bacillus circulans*,[2,3] *Pseudomonas fluorescens,*[4] *Serratia marcescens,*[5] *Escherichia coli*,[6,7] *Erwinia carotovora,*[8] *Proteus vulgaris,*[9] fungi *Aspergillus*, *Penicillium* and *Fusarium*,[10–14] yeast strains (*Saccharomyces cerevisiae)* and actinomycetes (*Streptomyces karnatakensis, Streptomyces venezuelae)* having the potential of L-asparaginase enzyme production, have been isolated from various xenobiotic sources and characterized for their growth-related enzyme production pattern along with enzymatic properties for their clinical, economic and immunological suitability. For industrial use, high-level production and process economics are the most important aspects to be considered. It was noticed that microbial growth, metabolism and product production is influenced by fermentation related nutritional and physiological parameters.[14–18] Hence, process optimization became of central importance and an undisputed component in industrial production processes especially with regard to biotechnological production processes, in which even small improve-

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ments can be decisive for commercial success. Optimization of different fermentation conditions can be achieved either by conventional or statistical methods. The conventional method follows the 'one at a time method' in which one variable is changed while all others are kept constant. This method is time-consuming and provides no information on interaction influences on overall productivity. Whereas, statistical methods are rapid and reliable, short lists significant nutrients, helps understand the interactions among the nutrients at various concentrations and reduces the total number of experiments tremendously.[19] Different types of statistical methods are developed for such optimization experiments.[20] Among them, the Plackett-Burman design (PBD)[21] helps in the identification of the most significant compounds and their concentrations from a large group for improving further optimization.

The Plackett-Burman design aims to select the most important variables in the system.[21] This design is practical especially when the investigator is faced with a large number of factors and is unsure which settings are likely to produce optimal or near optimal responses. The PB design allows evaluation of $N - 1$ variables using $N$ number of experiments ($N$ must be a multiple of four). Each variable is represented at two levels namely, "high"(+) and "low"(–). These levels define the upper and lower limits of the range covered by each variable. If there are not enough '$N - 1$' variables, one can use dummy variables. In some cases, during the analysis of the results no degrees of freedom are left to estimate the error variability so one may not get the standardized effects and other ANOVA factors like $F$ and $p$ –values; this situation is normally called a saturated PBD. To avoid this situation, most of the authors use dummy variables to perform the additional number of experiments[22–25] which may increase the cost of experimentation. Keeping this in view, in the present study an effort has been made to describe how to obtain the standardized effects from the saturated design without performing the additional experiments, thus economizing the optimization process. This was exemplified by investigating the importance of metaboliomics related nutritional compounds that influence L-asparaginase production in isolated *Bacillus circulans* (MTCC 8574). The data indicated that all selected nutritional sources could be grouped into significant, non-significant, and most significant. This character of each nutritional source is observed to be dependent on the selected concentration. Overall, the study revealed more than a 200 % improvement in L-asparaginase enzyme production with this isolated *Bacillus* sp. upon optimization using saturated PBD without dummy variables.

## Materials and methods

### Microorganism and growth conditions

Isolated *Bacillus circulans* (MTCC 8574) was used for this study and the culture was stored on M9 medium slants. The medium consisted of (g $L^{-1}$): $Na_2HPO_4$, 6.0; $KH_2PO_4$, 3; NaCl, 0.5; $CaCl_2$, 0.011; $MgSO_4 \cdot 7H_2O$, 0.12; L-asparagine, 5.0 having pH 7.0. The culture was sub-cultured at every 7 days interval.

### L-Asparaginase production by submerged fermentation (SMF)

Fermentation experiments were performed in a 250 mL conical flask containing 100 mL of sterile medium (pH 7.0) consisting of (g $L^{-1}$): $Na_2HPO_4$, 6.0; $K_2HPO_4$, 3; NaCl, 0.5; L-asparagine, 5; $CaCl_2$, 0.011; $MgSO_4 \cdot 7H_2O$, 0.12. Two percent inoculum having 0.8 absorbance at $\lambda = 600$ nm was inoculated under sterile conditions and incubated at 37 °C in an incubator cum shaker. After 24 hours, the samples were collected and centrifuged at 5000 rpm. The supernatant was used as the enzyme source for measurement of enzyme activity and the pellet was used for the estimation of biomass. The selected different carbon sources (lactose, fructose, glucose, ribose, arabinose, maltose, sucrose, mannose, starch, galactose and xylose), and nitrogen sources (peptone, urea, potassium nitrate, ammonium sulphate, yeast extract, ammonium chloride, beef extract, tryptone, sodium glutamate, corn steep liquor, sodium nitrate, casein, ammonium nitrate, gelatin and proline) were supplemented at predetermined concentration and as per experimental design before sterilization of the medium to study their significance on L-asparaginase activity.

### Estimation of L-asparaginase activity

L-Asparaginase enzyme assay was measured by colorimetric method, according to Wriston and Yellin[26] at 37 °C, using CECIL UV-visible spectrophotometer, by estimating the ammonia produced during L-asparagine hydrolysis using Nessler's reagent. Reaction mixture consisted of 0.5 mL of 0.08 mmol $L^{-1}$ L-asparagine, 1.0 mL of 0.05 mmol $L^{-1}$ borate buffer (pH 7.5) and 0.5 mL of enzyme solution. The reaction was terminated by the addition of 0.5 mL of 15 % trichloroacetic acid solution. The liberated ammonia was coupled with Nessler's reagent, and quantitatively determined using standard curve. One unit of the L-asparaginase (IU) is defined as the amount of enzyme capable of producing 1 μmol of ammonia per minute at assay conditions.

### Plackett-Burman design

Two different PB Designs (one for analysis of carbon sources and the other for nitrogen sources) were used for analysis of the experimental results.

Analysis of the experimental results was performed based on the first order model to calculate the coefficient value of each selected constituent using the following eq. (1).

$$Y = \beta_0 + \Sigma\beta_i x_i \text{ (i = 1,2,3------k)} \quad (1)$$

Where:

$Y$ – response (productivity),

$\beta_0$ – model intercept,

$\beta_i$ – variables estimates.

A high coefficient value, either positive or negative, indicates that the analyzed factor has a major impact on production titer; while a coefficient value close to zero suggests a little or no impact. In contrast, a low *p*-value (probability value) indicates a "real" or significant effect.

The effect of each selected variable on L-asparaginase production was determined using eq. (2).

$$E(x_i) = \frac{2(\Sigma Y_i^+ - Y_i^-)}{N} \quad (2)$$

Where

$E(x_i)$ – concentration effect of the tested variable

$Y_i^+$ and $Y_i^-$ – L-asparaginase production from the trials where the variable ($x_i$) was measured at high and low concentrations, respectively;

$N$ – number of trials.

The sign of the effect indicates the level at which it is considered for further improvement. For example, a negative sign in front of a variable 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 L-asparaginase productivity was taken as responses ($Y$). The variables with confidence levels higher than 90 % were considered for L-asparaginase production.

## Results and discussion

In the present investigation, the significance of 11 ($k$ = 11) different carbon and 15 ($k$ = 15) different nitrogen sources on L-asparaginase production using *Bacillus circulans* was screened in order to improve the composition of the medium by simultaneous comparisons between two levels (high and low values) of the above selected factors by applying 12 (Table 1) and 16 (Table 2) experimental

Table 1 – *The Plackett-Burman experimental design matrix for screening of carbon compounds for L-asparaginase production by Bacillus circulans*

| Trial no $N$ | | Lactose | Fructose | Glucose | Ribose | Arabinose | Maltose | Sucrose | Mannose | Starch | Galactose | Xylose | L-Asparaginase activity (U mL$^{-1}$) | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | '–' level (g L$^{-1}$) | 0.5 | 1.0 | 1.5 | 1.5 | 1.5 | 1.5 | 1.0 | 0.5 | 1.0 | 1.0 | 0.5 | observed | predicted | error |
| | '+' level (g L$^{-1}$) | 1.5 | 2.0 | 3.0 | 3.0 | 3.0 | 3.0 | 2.0 | 1.5 | 2.0 | 2.0 | 1.5 | | | |
| 1 | | – | + | – | + | – | + | + | – | + | – | + | 57.95 | 59.69 | –1.74 |
| 2 | | – | – | + | + | + | + | – | + | + | – | – | 0.62 | 0.68 | –0.06 |
| 3 | | – | + | + | – | + | – | – | – | + | + | + | 18.35 | 18.28 | 0.064 |
| 4 | | – | – | – | + | + | – | + | + | – | + | + | 0.32 | 1.13 | –0.80 |
| 5 | | + | + | – | – | + | + | – | + | – | – | + | 28.97 | 30.71 | –1.74 |
| 6 | | + | – | – | – | + | + | + | – | + | + | – | 105.27 | 103.53 | 1.74 |
| 7 | | + | – | + | + | – | + | – | – | – | + | + | 18.35 | 19.15 | –0.8 |
| 8 | | + | – | + | – | – | – | + | + | + | – | + | 17.38 | 19.99 | –2.61 |
| 9 | | – | – | – | – | – | – | – | – | – | – | – | 28.97 | 29.03 | –0.06 |
| 10 | | + | + | – | + | – | – | – | + | + | + | – | 19.31 | 16.70 | 2.61 |
| 11 | | + | + | + | + | + | – | + | – | – | – | – | 9.65 | 8.85 | 0.80 |
| 12 | | – | – | + | – | – | + | + | + | – | + | – | 0.96 | –1.64 | 2.61 |

Table 2 – *The Plackett-Burman experimental design matrix for screening of nitrogen compounds for L-asparaginase production by Bacillus circulans*

| Trial no *N* | '–' level (g $L^{-1}$) / '+' level (g $L^{-1}$) | Peptone | Urea | P.N | A.S | Yeast extract | A.C | Beef extract | Tryptone | S.G | CSL | S.N | Casein | A.N | Gelatin | Proline | L-Asparagase activity (U $mL^{-1}$) | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | '–' level (g $L^{-1}$) | 1.5 | 1.5 | 5.0 | 5.0 | 1.5 | 5.0 | 1.5 | 1.5 | 1.5 | 1.5 | 5.0 | 1.5 | 5.0 | 1.5 | 1.5 | observed | predicted | error |
| | '+' level (g $L^{-1}$) | 3.0 | 3.0 | 10.0 | 10.0 | 3.0 | 10.0 | 3.0 | 3.0 | 3.0 | 3.0 | 10.0 | 3.0 | 10.0 | 3.0 | 3.0 | | | |
| 1 | | + | + | – | + | + | – | + | – | + | – | – | + | – | – | – | 22.21 | 22.70 | –0.49 |
| 2 | | – | – | – | + | + | + | – | + | – | – | + | + | + | + | – | 82.67 | 83.17 | –0.49 |
| 3 | | + | – | – | – | – | – | – | + | + | + | + | + | + | – | – | 150.86 | 146.47 | 4.39 |
| 4 | | + | – | + | + | – | + | + | – | – | + | – | – | + | – | – | 149.41 | 145.14 | 4.27 |
| 5 | | – | + | + | + | – | – | – | + | + | + | – | – | – | + | – | 50.41 | 46.14 | 4.27 |
| 6 | | – | + | + | – | – | – | + | + | – | – | – | + | + | – | + | 114.55 | 114.17 | 0.37 |
| 7 | | + | – | + | – | – | + | – | – | + | – | – | + | – | + | + | 113.58 | 113.21 | 0.37 |
| 8 | | + | – | – | + | – | – | + | + | – | – | + | – | – | + | + | 15.64 | 15.15 | 0.49 |
| 9 | | + | + | – | – | + | – | – | – | – | + | – | – | + | + | + | 50.31 | 54.71 | –4.3 |
| 10 | | – | – | + | + | + | – | – | – | – | + | + | + | – | – | + | 46.94 | 51.21 | –4.27 |
| 11 | | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | 123. | 128.19 | –4.27 |
| 12 | | – | + | – | – | – | + | + | – | – | + | + | + | – | + | – | 69.34 | 64.95 | 4.39 |
| 13 | | – | – | – | – | + | + | + | + | + | + | – | – | – | – | + | 164.19 | 168.59 | –4.39 |
| 14 | | – | – | + | – | + | – | + | – | + | – | + | – | + | + | – | 67.51 | 67.88 | –0.37 |
| 15 | | – | + | – | + | – | + | – | – | + | – | + | – | + | – | + | 49.16 | 48.66 | 0.49 |
| 16 | | + | + | + | – | + | + | – | + | – | – | + | – | – | – | – | 99.09 | 99.46 | –0.37 |

Note: P.N = potassium nitrate, A.S = ammonium sulphate, A.C = ammonium chloride, B.E = beef extract, S.G = sodium glutamate, CSL = corn steep liquor, S.N = sodium nitrate and A.N = ammonium nitrate

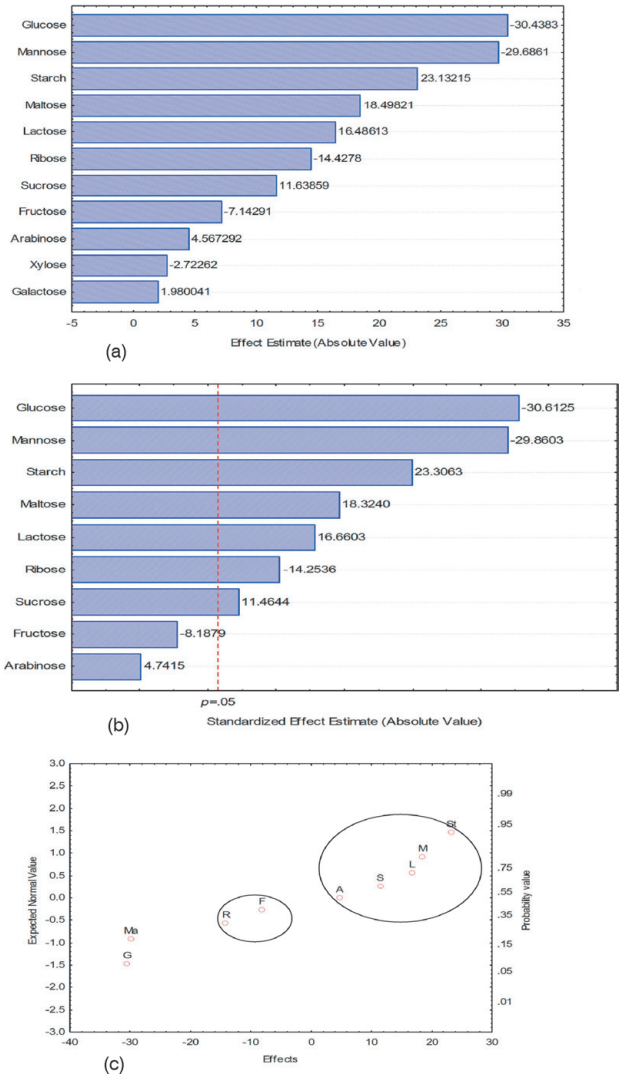
Plackett-Burman designs for carbon and nitrogen sources, respectively.

Among the selected different carbon sources, the highest activity (105.28 U $mL^{-1}$) was observed in the 6$^{th}$ trail run, whereas the least activity (0.325 U $mL^{-1}$) was observed in the 4$^{th}$ trail run (Table 1). In the case of nitrogen sources, the 13$^{th}$ and 8$^{th}$ experimental runs showed the highest and lowest L-asparaginase activity (164.19 and 15.64 U $mL^{-1}$), respectively (Table 2). Comparative evaluation of the observed lowest and highest enzyme production titers further suggests that nitrogen sources have more influence on *Bacillus circulans* metabolism related L-asparaginase production compared to carbon sources. Such carbon and nitrogen source mediated regulation of bacterial metabolism and product/enzyme production were also reported in the literature.[14–18,27] This could be evidenced based on the observation that the enzyme production was > 60 % higher with supplementation of nitrogen compared to carbon sources (Tables 1 and 2).

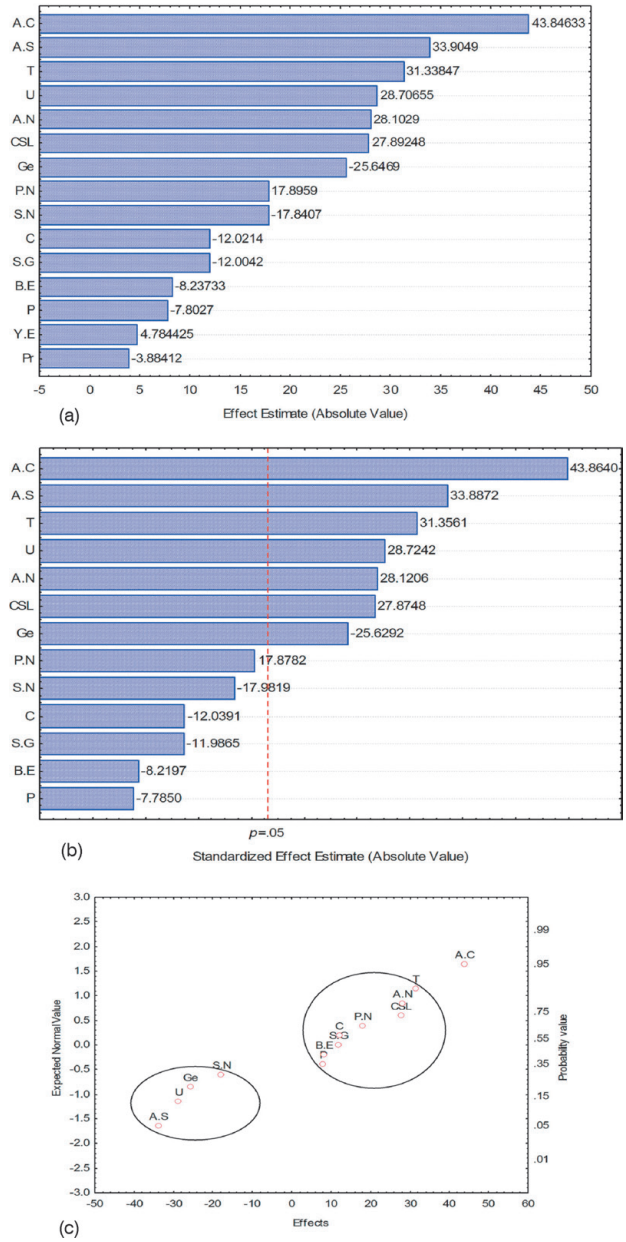
Using obtained responses (L-asparaginase activity in Tables 1 and 2), regression coefficients (11 parameters plus a constant term for the carbons source, and 15 parameters and 1 constant term for the nitrogen source) were calculated and the responses of each parameter of carbon and nitrogen sources were predicted using the multiple linear equations.

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

It is evident from the Pareto chart of carbon (Fig. 1a) that the most important carbon source for L-asparaginase production is glucose, followed by mannose. The third most important carbon source is




F i g . 1 – *Pareto chart for selected carbon source on* L*-asparginase production (a) Without pooling factors into error (real one) (b) After pooling two factors into error (c) Normal probability plot of chosen carbon source on* L*-asparginase production (L = lactose, F = fructose, G = glucose, R = ribose, A = arabinose, M = maltose, S = sucrose, Ma = mannose, St = starch, Ga = galactose and X = xylose)*




F i g . 2 – *Pareto chart for the selected nitrogen source on* L*-asparginase production (a) Without pooling factors into error (real one) (b) After pooling two factors into error (c) Normal probability plot of chosen nitrogen source on* L*-asparginase production (P = peptone, U = urea, P.N = potassium nitrate, A.S = ammonium sulphate, Y.E = yeast extract, A.C = ammonium chloride, B.E = beef extract, T = tryptone, S.G = sodium glutamate, CSL = corn steep liquor, S.N = sodium nitrate, C = casein, A.N = ammonium nitrate, Ge = gelatin, Pr = proline)*

starch. Lactose, fructose, ribose, arabinose, maltose and sucrose do not play an important role in the production of the L-asparaginase. The two most insignificant factors, as evidenced from the Pareto chart are xylose and galactose among selected carbon sources. Such carbon source dependent L-asparaginase enzyme production was reported in *Staphylococcus* sp.[19] Among different nitrogen sources studied, ammonium chloride denoted the highest effect on the L-asparaginase production while yeast extract and proline showed the lowest effect on the L-asparaginase production (Fig. 2a).

Further analysis of the Pareto chart of carbon (Fig. 1a) and nitrogen sources (Fig. 2a) denoted that none of the selected variables showed statistical significance on enzyme production. Though several reports are available in the literature on the use of PBD in bioproduct/enzyme production using different microbial strains, none of them denoted significance among selected carbon and nitrogen sources.[22–25]

To confirm further, analysis of variance (ANOVA) was done. In Tables 3 and 4, the $F$ statistics and $p$ values are not available, indicating that none of the

Table 3 – *Real and transformed ANOVA table for L-asparaginase production by Bacillus circulans in presence of selected carbon sources*

| | (a) ANOVA for the real design | | | | | (b) ANOVA with two main effects pooled into error | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | coefficient | *t*-value | SS | *F*-value | *p*-value | coefficient | *t*-value | SS | *F*-value | *p*-value |
| mean/interc. | 24.9179 | – | | – | – | 24.8308 | 21.2692 | | | 0.002203 |
| lactose | 8.2431 | – | 784.016 | – | – | 8.3301 | 7.1353 | 802.957 | 50.9128 | 0.019081 |
| fructose | –3.5715 | – | 106.294 | – | – | –4.0940 | –3.0927 | 150.846 | 9.5646 | 0.090572 |
| glucose | –15.2192 | – | 2672.576 | – | – | –15.3063 | –13.11 | 2710.972 | 171.8935 | 0.005767 |
| ribose | –7.2139 | – | 600.466 | – | – | –7.1268 | –6.1046 | 587.731 | 37.266 | 0.0258 |
| arabinose | 2.2836 | – | 60.174 | – | – | 2.3707 | 2.0307 | 65.036 | 4.1237 | 0.179391 |
| maltose | 9.2491 | – | 987.068 | – | – | 9.1620 | 7.8479 | 971.335 | 61.5891 | 0.015852 |
| sucrose | 5.8193 | – | 390.741 | – | – | 5.7322 | 4.91 | 380.216 | 24.1082 | 0.039065 |
| mannose | –14.8430 | – | 2542.108 | – | – | –14.9301 | –12.788 | 2579.374 | 163.5493 | 0.006059 |
| starch | 11.5661 | – | 1543.547 | – | – | 11.6532 | 9.9817 | 1571.355 | 99.6343 | 0.009888 |
| galactose | 0.9900 | – | 11.309 | – | – | | | | | |
| xylose | –1.3613 | – | 21.383 | – | – | | | | | |
| error | | | 0 | | | | | 31.542 | | |
| total SS | | | 9753.569 | | | | | 9753.569 | | |

SS = Sum of squares

Table 4 – *Real and transformed ANOVA table for L-asparaginase production by Bacillus circulans in presence of selected nitrogen sources*

| | (a) ANOVA for the real design | | | | | (b) ANOVA with two main effects pooled into error | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | coefficient | *t*-value | SS | *F*-value | *p*-value | coefficient | *t*-value | SS | *F*-value | *p*-value |
| mean/interc. | 86.7319 | – | | – | – | 86.7408 | 39.43497 | | | 0.0006 |
| peptone | –3.9014 | – | 238.66 | – | – | –3.8925 | 1.76966 | 237.77 | 3.1317 | 0.2188 |
| urea | 14.3533 | – | 3230.34 | – | – | 14.3621 | –6.52945 | 3236.85 | 42.6336 | 0.0226 |
| potassium nitrate | 8.9480 | – | 1255.43 | – | – | 8.9391 | 4.06399 | 1253.94 | 16.5160 | 0.0555 |
| ammonium sulphate | 16.9525 | – | 4506.21 | – | – | 16.9436 | –7.70308 | 4505.05 | 59.3375 | 0.0164 |
| yeast extract | 2.3922 | – | 89.73 | – | – | | | | | |
| ammonium chloride | 21.9232 | – | 7536.20 | – | – | 21.9320 | 9.97094 | 7548.19 | 99.4197 | 0.0099 |
| beef extract | –4.1187 | – | 265.99 | – | – | –4.1098 | 1.86846 | 265.06 | 3.4911 | 0.2026 |
| tryptone | 15.6692 | – | 3849.83 | – | – | 15.6781 | 7.12772 | 3857.20 | 50.8044 | 0.0191 |
| sodium glutamate | –6.0021 | – | 564.87 | – | – | –5.9933 | 2.72472 | 563.65 | 7.4240 | 0.1124 |
| corn steep liquor | 13.9462 | – | 3049.72 | – | – | 13.9374 | 6.33637 | 3048.25 | 40.1495 | 0.0240 |
| sodium nitrate | –8.9203 | – | 974.77 | – | – | –8.9910 | –3.68448 | 1030.68 | 13.5754 | 0.0664 |
| casein | –6.0107 | – | 566.50 | – | – | –6.0195 | 2.73666 | 568.61 | 7.4893 | 0.1116 |
| ammonium nitrate | 14.0515 | – | 3095.91 | – | – | 14.0603 | 6.39222 | 3102.23 | 40.8605 | 0.0236 |
| gelatin | –12.8234 | – | 2578.42 | – | – | –12.8146 | –5.82591 | 2576.90 | 33.9411 | 0.0282 |
| proline | –1.9421 | – | 59.14 | – | – | | | | | |
| error | | | 0.00 | – | | | 2 | 75.92 | | |
| total SS | | | 32596.95 | – | | | | 32596.95 | | |

SS = Sum of squares

selected carbon and nitrogen sources has an impact on L-asparaginase production. This may have occurred because no degree of freedom was left to estimate the error variability and hence, the plot for standardized effect is not produced. Such type of saturated PBD experimental condition was not reported in biotechnological applications; however, the saturated PBD was described by Ryan,[28] Deming and Morgan.[20] To obtain the standardized effects, the *F* and *p* values from saturated PBD, the most insignificant two factors from each group based on the Pareto chart were ignored. The least significant factors, among selected carbon sources were xylose and galactose, and among nitrogen sources yeast extract and proline were selected for pooling their effects on L-asparaginase production into error. Accordingly, the transformed ANOVA data are represented in Table 3b for carbon sources and Table 4b for nitrogen sources.

Analysis of the transformed carbon source ANOVA data (Table 3b) indicated that fructose and arabinose are also insignificant for the L-asparaginase production among the selected carbon sources in this bacterial strain. The observed lowest *p*-value (0.0057) for glucose indeed suggested that this carbon source is the most important nutrient for the L-asparaginase production. Contradicting reports are available in the literature on glucose mediated L-asparaginase production in different microbial strains. Inducing role of glucose on L-asparaginase production was reported in *Serratia marcescens*[29] and in *E. coli*.[30] Dejong[31] reported that glucose works as repressor rather than inducer of L-asparaginase production in *Streptomyces griseus*. However, glucose concentration dependent repression functionality was observed in *Serratia marcescens*, where supplementation of 0.4 % glucose caused a marginal reduction and when its concentration increased to 1.0 %, which resulted in a 35 % reduction on L-asparaginase production.[32] Mannose (*p*-value 0.006) was found to be the next significant carbon source influencing the L-asparaginase production in this *Bacillus circulans* strain after glucose. This is further evidenced from the pareto chart generated for different carbon sources (Fig. 1b). Such data also revealed that the selected microbial strain differs in its metabolism-mediated L-asparaginase production pattern compared to literature reported strains.

Fig. 2b depicted that urea, ammonium sulphate, ammonium chloride, tryptone, ammonium nitrate and gelatin are the significant variables remaining and all are observed to be insignificant indicating that L-asparaginase production with this microorganism is more favorable with supplementation of selected inorganic nitrogen source rather than the organic nitrogen source, except tryptone and gelatin. It is interesting to note that none of the complex nitrogen sources showed a significant influence on enzyme production. The observed reduction in enzyme production in this bacterial strain may be attributed to concomitant production of protease similar to the literature report on L-asparaginase production in fungal strains.[14] This data is in contrast with the observed enhanced L-asparaginase production in the presence of corn steep liquor and casein by *Pseudomonas aeruginosa*.[33] Whereas yeast extract mediated enhanced enzyme production was reported by Sukumaran *et al*.[29] and Liu and Zajic,[34] in *Serratia marcescens* and *Erwinia aroideae*, respectively. Proline as nitrogen source was reported as an inducer of the L-asparaginase enzyme in *Aspergillus terreus* and *Aspergillus tamari*[14] while the same compound was observed as insignificant in the present study (Fig. 2b). Among significant selected nitrogenous compounds, ammonium chloride has the highest significance followed by ammonium sulphate.

The probability plot of effects is very useful for separating random noise from 'real' effects based on their distribution on the plot. It is evident from Fig. 1c that among selected carbon sources, glucose and mannose were positioned as outliers with negative mean values and separated from other variables. Ribose and fructose revealed negative mean value while all other tested carbohydrates showed positive mean value. Even though glucose and mannose having the negative mean, these two are much far away from the ribose and fructose, so these two are the outliers in this graph which indicated that these two compounds have the more positive influence on the L-asparaginase production. In case of selected nitrogen sources, it was observed that ammonium chloride positioned as an outlier suggesting that ammonium chloride is the main nitrogen source for the L-asparaginase production (Fig. 2c). However, it is interesting to note that the outlier carbon sources showed a negative and nitrogen source indicated positive effect. This suggests that further manipulation of carbon- and nitrogen sources concentration improves the L-asparaginase production in this bacterial strain. Further experiments to enhance the L-asparaginase production and to study the impact of critical parameters at their individual and interactive level are in progress.

## Conclusions

The imperative role of different carbon and nitrogen sources and their concentrations on L-asparaginase production by *Bacillus circulans* MTCC 8574 has been investigated using Plackett-Burman program. Among tested sources, nitrogen sources

revealed more than 60 % regulatory role on L-asparaginase synthesis in this bacterial strain. Analysis of experimental data revealed no significance of tested nutrient components denoting the saturation in the design. In this presentation it is proved that such saturated data could be effectively used for finding out the significant factors such as '$p$' and '$t$' values by pooling the least significant parameters. This essentially plays an effective role in bioprocess optimization and understanding of process dynamics with respect to nutrient type and its concentration for microbial metabolite production during fermentation.

## List of symbols

$E$ – concentration effect of the tested variable
$N$ – number of trails
$X$ – variable measured
$Y$ – response
$\beta_0$ – intercept
$\beta_i$ – variables estimate
$\gamma$ – mass concentration, g $L^{-1}$
$\lambda$ – wavelength, nm

## References


1. *Broome, J. D.,* Nature **191** (1961) 1114.
2. *Prakasham, R. S., Hymavathi, M., Subba Rao, Ch., Arepalli, S. K., Venkateshwar Rao, J., Kavin Kennady, P., Nasaruddin, K., Vijay Kumar, J. B., Sarma, P. N.,* Appl. Biochem. Biotechnol. **160** (2010) 72.
3. *Hymavathi, M., Sathish, T., Subba Rao, Ch., Prakasham, R. S.,* Appl. Biochem. Biotechnol. **159** (2009) 191.
4. *Degroot, N., Lichtenstein, N.,* Biochim. Biophys. Acta **40** (1960) 99.
5. *Rowley, B., Wriston, J. C.,* Biochem. Biophys. Res. Commun. **28** (1967) 160.
6. *Mashburn, L. T., Wriston, J. C.,* Arch. Biochem. Biophys. **105** (1964) 450.
7. *Kozak, M., Jurga, S.,* Acta Biochimica Polonica **49** (2002) **509.**
8. *Wade, H. E., Elsworth, R., Herbert, D., Keppie, J.,* Sargeant, K., Lancet II **2** (1968) 776.
9. *Tosa, T., Sano, R., Yamamoto, K., Nakamura, M., Ando, K., Chibata, I.,* Applied Microbiol. **22** (1971) 387.
10. *Curran, M. P., Daniel, R. M., Guy, R. G., Morgan, H. W.,* Arch. Biochem. Biophys. **241** (1985) 571.
11. *Gulati, R., Saxena, K., Gupta, R.,* Lett. Appl. Microbiol. **24** (1997) 23.
12. *Boss, J.,* Int. J. Clin. Pharm. Therap. **35** (1997) 96.
13. *Gallagher, M. P., Marshall, R. D., Wilson, R.,* Essays Biochemistry **24** (1999) 1.
14. *Sarquis, M. I., Oliveira, E. M., Santos, A. S., Costa, G.,* Mem. Inst. Oswaldo Cruz **99** (2004) 489.
15. *Greasham, R. L.,* Media for Microbial Fermentations, in *Rehm, H. J., Reed, G., Puhler, A., Stadler, P. J. W.* ( Eds.), Bioprocessing. In: Biotechnology, Vol. 3, VCH Publi., New York, 1983, pp 128–139.
16. *Srinivasulu, B., Prakasham, R. S., Jetty, A., Srinivas, S., Ellaiah, P., Ramakrishna, S. V.,* Process Biochem. **38** (2002) 593.
17. *Sreenivas Rao, R., Prakasham, R. S., Prasad, K. K., Rajesham, S., Sarma, P. N., Rao, L. V.,* Process Biochem. **39** (2004) 951.
18. *Prakasham, R. S., Subba Rao, Ch., Rao, R. S., Rajesham, S., Sarma, P. N.,* Applied Biochem. Biotechnol. **121** (2005) 133.
19. *Prakasham, R. S., Subba Rao, Ch., Rao, R. S., Sarma, P. N.,* J. Applied Microbiol. **102** (2007) 1382.
20. *Deming, S. N., Morgan, S. L.,* Experimental Design A Chemometric Approach, Elsevier Publ., Oxford, 1987.
21. *Plackett, R. L., Burman, J. P.,* Biometrika **33** (1964) 305.
22. *Kishor, C., Ujjval, T., Kamlesh, C. P.,* Bioresource Technol. **98** (2007) 98.
23. *Reddy, L. V. A., Wee, Y., Yun, J., Ryu, H.,* Bioresource Technol. **99** (2008) 2242.
24. *Levina, L., Forchiassina, F., Vialeb, A.,* Process Biochem. **40** (2005) 1381.
25. *Pan, C. M., Fan, Y. T., Xing, Y., Hou, H. W., Zhang, M. L.,* Bioresource Technol. **99** (2008) 3146.
26. *Wriston, J. C., Yellin, T. O.,* Adv. Enzymol. **39** (1973) 185.
27. *Subba Rao, Ch., Sathish, T., Mahalaxmi, M., Laxmi, G. S., Rao, R. S., Prakasham, R. S.,* J. Applied Microbiol. **104** (2008) 889.
28. *Ryan, T. P.,* Statistical Methods for Quality Improvement. Wiley Publ., Newyork, 1989.
29. *Sukumaran, C. P., Singh, D. V., Mahadevan, P. R.,* J. Biosci. **1** (1979) 263.
30. *Boeck, L. D., Squires, R. W., Wilson, M. W., Ho, P. P. K.,* Appl. Microbiol. **20** (1970) 964.
31. *Dejong, P. J.,* Appl. Microbiol. **23** (1972) 1163.
32. *Heinemann, B., Howard, A. J.,* Appl. Microbiol. **18** (1969) 550.
33. *Abdel-Fattah, Y. R., Olama, Z. A.,* Process Biochem. **38** (2002) 115.
34. *Liu, F. S., Zajic, J. E.,* Appl. Microbiol. **25** (1973) 92.