Research Article

# Optimization of Carbon and Nitrogen Substrate for Alkaline Phosphatase Yields of A Halotolerant Facultative Alkaliphile *Bacillus flexus* FPB17 Using Response Surface Methodology

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**ABSTRACT**

Studies on the optimization of yields of alkaline phosphatase from a halotolerant facultative alkaliphile *Bacillus flexus* FPB17 from North Gujarat Lake system sediments using response surface methodology for assessing interactive effects of Carbon and Nitrogen substrates has resulted in 3.57 fold enhancement of alkaline phosphatase yields and development of a simple and less time consuming experimental design. Effect of N-sources *viz.* $NH_4Cl$, $(NH_4)_2SO_4$, $(NH_4)_2NO_3$, meat extract, peptone, tryptone, urea, yeast extract and their combinations in arbitrarily selected concentrations without C-source was first evaluated, followed by the impact of pairing of significant N-sources with different C-sources *viz.* glucose, fructose, ribose, sucrose, maltose, mannitol, starch, sodium acetate, cellulose, sorbitol in 1:1 and 2:1 C:N ratio. The effect of cheap N-sources like Corn steep liquor and Soybean flour and cheap C-sources like molasses and whey was also checked. The concentration of best C and N-source combination (ribose, meat extract and peptone) was later optimized by using Central Composite Design obtaining 1270.21 U/ml alkaline phosphatase. The experimental alkaline phosphatase yields matched with the predicted yields confirming the soundness of the experimental design. The enzyme production was non-growth associated and C-sources exhibited carbon catabolite repression.

**Keywords:** *Bacillus flexus* FPB17, Central Composite Design, Carbon catabolite repression, Halotolerant facultative alkaliphile, Response surface methodology.

## INTRODUCTION

Alkaline phosphatases (ALPs; EC 3.1.3.1) are metalloenzymes existing mostly as metal ion binding dimers, in a large biodiversity ranging from bacteria to man.[1, 2] These catalyze nonspecific hydrolysis of phosphate monoesters into inorganic phosphate and alcohol. The optimization of various carbon, nitrogen and other nutrients and fermentation parameters is an essential pre-requisite for exploiting the genetic potential of the strain and for developing a cost-effective fermentation process for a microbial metabolite. This helps to maintain a balanced nutrition and minimizes the unutilized components.[3-5] The conventional methods involving the variations of one parameter at a time and keeping the others constant are not only time consuming but expensive as well. These have lately been replaced by factorial design and regression analysis and response surface methodology even for microbial enzymes.[6-15] The 3D and counter plots for response surfaces can provide a good way for visualizing the parameter interactions and for predicting optimum process conditions for microbial enzyme production. There are no such documented systematic studies on ALP production from *Bacillus flexus*. Considering the several emerging commercial applications of ALP, the optimization and enhancing of yields of extracellular ALP from *Bacillus flexus* FPB17 was undertaken using central composite design for assessing interactive effects of Carbon and Nitrogen substrates and for developing a simpler and less time consuming experimental design, using response surface methodology.

## MATERIALS AND METHODS

### Culture & production medium conditions

*Bacillus flexus* FPB17, used in the study, is an isolate from the alkaline lake sediments from Bhilot village, of Patan in North Gujarat. The strain was maintained on nutrient agar, inoculum preparation was carried out in 25 ml nutrient broth containing 1% peptone, 1% meat extract, 0.5% NaCl with initial pH 9.0 in 100 ml Erlenmeyer flask, incubated on an orbital shaker at 35°C and 120 rpm for 6-8 h achieving 0.8-1.2 O.D. at 600 nm. 2% v/v inoculum was transferred to 50 ml of fermentation medium 1% peptone, 1% meat extract, 0.5% NaCl, 0.05% $MgCl_2$, 0.05% $CaCl_2$, 0.01% KCl, 0.007% $KH_2PO_4$ with initial pH 9.0 in 250 ml Erlenmeyer flask and incubated at 35°C, 120 rpm for 48 h.

### Analysis of ALP

ALP activity was measured spectrophotometrically by determining the release of *p*-nitrophenol (*p*-NP) from *p*-nitrophenyl phosphate disodium salt (*p*-NPP) at 400 nm.[16, 17] 100 µl cell free supernatant was added to 1000 µl of *p*-NPP solution (1.35 mM in 50 mM Tris-HCl buffer at pH 9.0) and the mixture was incubated at 35°C for 10 minute. The absorbance of the product was measured at 400 nm using UV-Vis spectrophotometer. One unit of enzyme activity is the amount of ALP required for catalyzing the liberation of 1µmol of *p*-NP per min.

### C and N substrates

The effect of incorporation of different N-sources *viz.* $NH_4Cl$, $(NH_4)_2SO_4$, $(NH_4)_2NO_3$, meat extract, peptone, tryptone, urea, yeast extract, and their combinations on ALP production was studied arbitrarily first. This was followed by pairing of significant N-sources with different C-sources *viz.* sodium acetate, cellulose, fructose, glucose, maltose, mannitol, ribose, sucrose, starch, sorbitol in 1:1 and 2:1 C:N ratio. The effect of cheap N-sources like Corn steep liquor (CSL) and Soya flour and cheap C-sources like molasses and whey was also checked on the production of ALP. The selected C and N-source combination was optimized by using Central composite design (CCD). CCD was applied to determine the optimum concentration of the significant C and N-sources screened arbitrarily. The design with six start points and six replicates at the central point, resulting in 20 experiments was generated by Design Expert, Version 8.0.6.1, Stat-Ease Inc., Minneapolis (MN) statistical software. The levels of factors used for experimental design are given in Table 1 and the coded variables are calculated according to the equation 1:

$$X_i = \frac{(X_i - X_i^x)}{\Delta X_i} \quad i = 1, 2, 3, \ldots\ldots\ldots, j \quad --- \text{equation 1}$$

The behaviour of the system was explained by second order polynomial equation 2:

$$Y = b_0 + \sum_i b_i X_i + \sum_i b_{ij} X_i X_j + \sum b_{ij} X_i^2 + e \quad --\text{equation 2}$$

Where Y is the predicted response, $X_i$, $X_j$ are input variables which influence the response variable Y; $b_0$ is the offset term; $b_i$ is the $i^{th}$ linear coefficient; $b_{ii}$ is the $i^{th}$ quadratic coefficient and $b_{ij}$ is the $ij^{th}$ interaction coefficient. Analysis of variance (ANOVA) and regression analysis were done and contour plots were drawn by using Design Expert, Version 8.0.6.1.

## RESULTS AND DISCUSSION

### Effect of different N-sources and their combinations on ALP production by *Bacillus flexus* FPB17

The different N-sources: $NH_4Cl$, $(NH_4)_2SO_4$, $(NH_4)_2NO_3$, meat extract, peptone, tryptone, urea, yeast extract (all at 2% level) and their combinations apart from cheap N-sources: CSL and soya flour were incorporated in fermentation medium without any C-source, to study their impact on extracellular ALP yields. The results presented in Table 1 indicate maximum enzyme activity (377.08 U/ml) was exhibited by medium with meat extract (1%) and peptone (1%), followed by medium with 2% meat extract (225.00 U/ml), while the optimum growth of the strain was obtained in medium with 2% yeast extract. It is surprising that soybean flour / CSL, the commonly used cheap industrial N-sources were not good enough for ALP yields by this strain. The animal protein based N-substrates proved more suitable. The best growth was observed with yeast extract (2 g%). No correlation between the growth and ALP yields was observed.

Neddermann and Nausch, 2004[18] checked the effects of organic and inorganic N-compounds on the activity of bacterial ALP and reported that addition of certain N-sources to the medium enhanced the ALP production to a high degree, while the addition of the amino acid leucine and of inorganic nitrogen alone resulted in an inhibition of the bacterial ALP. The lower medium yields of ALP in soya flour containing medium is explainable by the fact that soya flour contains very high amount of leucine (109 g / 100 g of protein) as well as isoleucine (115 g / 100 g of protein) as reported by Ali *et al.*, 2009.[19]

Same results obtained with *Bacillus flexus* where all the inorganic N-sources alone and the cheap N-source soya flour (due to its higher leucine content) inhibited the ALP activity. Lower performance of CSL can be due to inhibitory effect of its 2.5% Glucose content[20] on ALP from *Bacillus flexus.*

**Table 1:** Effect of different N-sources and their combinations on ALP yields by Bacillus flexus FPB17 in absence of C –sources

| Nitrogen Source (2 g%) | ALP activity (U/ml) | Growth (O.D.at 600 nm) |
|---|---|---|
| $NH_4Cl$ | 18.75 ± 0.1 | 0.007 |
| $(NH_4)_2NO_3$ | 19.44 ± 0.1 | 0.005 |
| $(NH4)_2SO_4$ | 19.44 ± 0.1 | 0.005 |
| Meat extract | 225.00 ± 0.2 | 1.965 |
| Meat extract (1 g%) + peptone (1 g%) | 377.08 ± 0.3 | 1.676 |
| Meat extract (1 g%) + tryptone (1 g%) | 203.47 ± 0.2 | 2.098 |
| Peptone | 4.17± 0.2 | 1.927 |
| Tryptone | 87.50 ± 0.5 | 1.650 |
| Urea | 20.83 ± 0.1 | 0.065 |
| Yeast extract | 20.83 ± 0.2 | 2.140 |
| Yeast extract (1 g%) + peptone (1 g%) | 75.69 ± 0.3 | 1.765 |
| Yeast extract (1 g%) + tryptone (1 g%) | 127.78 ± 0.0 | 2.115 |
| Corn steep liquor | 146.13 ± 0.2 | 1.980 |
| Soya flour | 167.21 ± 0.1 | 2.120 |

### Effect of different C-sources and their combinations with selected N-source on ALP production by *Bacillus flexus* FPB17

Initially 2 g% of different C-sources (glucose, fructose, ribose, sucrose, maltose, mannitol, starch, sodium acetate, cellulose, sorbitol) as well as cheap C-sources molasses and whey were incorporated in *Bacillus flexus* fermentation medium with the best N-source from above experiments (meat extract 1 g% and peptone 1 g%) keeping C:N ratio of 1:1. Maximum ALP activity (186.11

U/ml) was obtained in the fermentation medium having only N-source, while the media containing 2 g% C-sources showed very low ALP yields (Table 2). The best growth was observed with ribose and fructose. This proved that N-source alone was sufficient for good ALP activity and C-sources exhibited carbon catabolite repression here. No correlation between the growth and ALP yields was observed. These results are in line with the observations of Bandyopadhyay and Majumdar, 1974[21] who reported glucose, maltose, dextrin, starch and mannose being inhibitory to ALP production by *Streptomyces fradiae*. 1% glucose and 0.5% pyruvate have, however, been reported to stimulate the ALP production in *Bacillus intermedius*.[22]

C-source and pH of the medium were reported to be influencing the ALPs in *Neurospora crassa*.[23]

The change of C:N ratio of 1:1 by taking 2 g% C-sources and meat extract 0.5 g% and peptone 0.5 g%, the maximum ALP activity (406.94 U/ml) achieved in the medium containing 2 g% ribose and next higher in control, sodium acetate and whey, respectively (Table 2). The reduction of nitrogen resulted in improved yields of ALP in media containing sodium acetate, fructose and whey. Once again the enzyme production was non-growth associated. The commonly used cheap industrial C-sources molasses and whey resulted in low ALP yields, the whey being little better at both C:N ratios.

**Table 2:** Effect of various C-sources on ALP yields by *Bacillus flexus* FPB17 at 1:1 and 2:1 C:N ratio in medium having meat extract (1 g%) + peptone (1 g%) and meat extract (0.5 g%) + peptone (0.5 g%) respectively.

| Carbon Source (2 g%) | 1:1 C:N ratio | | 2:1 C:N ratio | |
|---|---|---|---|---|
| | ALP activity (U/ml) | Growth (O.D.at 600nm) | ALP activity (U/ml) | Growth (O.D.at 600nm) |
| Control | 186.11 ± 0.0 | 1.88 | 186.11 ± 0.0 | 1.88 |
| Sodium acetate | 84.03 ± 0.1 | 2.07 | 165.28 ± 0.2 | 2.78 |
| Cellulose | 73.61 ± 0.3 | 1.90 | 25.00 ± 0.3 | 2.58 |
| Fructose | 42.36 ± 0.1 | 2.18 | 97.22 ± 0.2 | 2.41 |
| Glucose | 25.00 ± 0.2 | 1.40 | 16.67 ± 0.0 | 1.78 |
| Maltose | 25.69 ± 0.2 | 1.46 | 20.14 ± 0.1 | 1.76 |
| Mannitol | 31.94 ± 0.3 | 1.93 | 30.56 ± 0.3 | 2.34 |
| Ribose | 43.06 ± 0.1 | 2.20 | 406.94 ± 0.5 | 2.35 |
| Sorbitol | 16.14 ± 0.2 | 1.49 | 20.14 ± 0.3 | 1.89 |
| Starch | 25.00 ± 0.1 | 1.50 | 31.94 ± 0.2 | 1.87 |
| Sucrose | 24.31 ± 0.2 | 1.26 | 13.19 ± 0.4 | 1.58 |
| Molasses | 32.57 ± 0.2 | 1.45 | 16.27 ± 0.2 | 1.26 |
| Whey | 72.48 ± 0.4 | 1.97 | 148.23 ± 0.3 | 2.12 |

**Table 3:** ALP yields by *Bacillus flexus* using significant C and N-sources based on CCD criterion

| Run order | Point type | A: Ribose g% | B: Meat extract g% | C: Peptone g% | Unit activity of ALP | | |
|---|---|---|---|---|---|---|---|
| | | | | | Experimental | Predicted | Residual |
| 1 | Center | 2.00 | 1.00 | 1.00 | 1015.32 | 1035.40 | -20.08 |
| 2 | Factorial | 4.00 | 0.00 | 2.00 | 765.24 | 781.45 | -16.21 |
| 3 | Axial | 2.00 | 1.00 | 2.68 | 635.78 | 631.89 | 3.89 |
| 4 | Center | 2.00 | 1.00 | 1.00 | 1014.00 | 1035.40 | -21.40 |
| 5 | Center | 2.00 | 1.00 | 1.00 | 1016.02 | 1035.40 | -19.38 |
| 6 | Factorial | 4.00 | 2.00 | 0.00 | 20.89 | 11.99 | 8.90 |
| 7 | Axial | 2.00 | 2.68 | 1.00 | 384.21 | 389.82 | -5.61 |
| 8 | Factorial | 0.00 | 0.00 | 2.00 | 267.12 | 277.57 | -10.45 |
| 9 | Factorial | 0.00 | 2.00 | 2.00 | 104.12 | 95.37 | 8.75 |
| 10 | Factorial | 4.00 | 0.00 | 0.00 | 242.31 | 252.61 | -10.30 |
| 11 | Center | 2.00 | 1.00 | 1.00 | 1015.84 | 1035.40 | -19.56 |
| 12 | Axial | 5.36 | 1.00 | 1.00 | 1.87 | -1.96 | 3.83 |
| 13 | Factorial | 0.00 | 2.00 | 0.00 | 128.84 | 114.17 | 14.67 |
| 14 | Axial | 1.36 | 1.00 | 1.00 | 914.28 | 960.26 | -45.98 |
| 15 | Center | 2.00 | 1.00 | 1.00 | 1014.78 | 1035.40 | -20.62 |
| 16 | Axial | 2.00 | 1.00 | 0.68 | 925.14 | 972.70 | -47.56 |
| 17 | Factorial | 0.00 | 0.00 | 0.00 | 5.89 | 11.41 | -5.52 |
| 18 | Center | 2.00 | 1.00 | 1.00 | 1016.12 | 1035.40 | -19.28 |
| 19 | Factorial | 4.00 | 2.00 | 2.00 | 259.84 | 255.86 | 3.98 |
| 20 | Axial | 2.00 | 0.68 | 1.00 | 1270.21 | 1052.29 | 217.92 |
| Control | – | 2.00 | 0.50 | 0.50 | 355.56 | – | – |

**Table 4:** Regression analysis of CCD criterion data for ALP production by *Bacillus flexus* FPB17

| Source | Coefficient | Standard Error of Coefficient | T Value | p-value |
|---|---|---|---|---|
| Constant | 1035.40 | 25.07 | 41.30 | 0.00 |
| Ribose | 100.42 | 25.15 | 3.99 | 0.00 |
| Meat Extract | -105.71 | 25.15 | -4.20 | 0.00 |
| Peptone | 127.51 | 25.15 | 5.07 | 0.00 |
| Ribose*Meat Extract | -85.85 | 26.28 | -3.27 | 0.01 |
| Ribose* Peptone | 65.67 | 26.28 | 2.50 | 0.03 |
| Meat Extract* Peptone | -71.24 | 26.28 | 2.71 | 0.02 |
| Ribose*Ribose | -426.47 | 27.45 | 15.54 | 0.00 |
| Meat Extract*Meat Extract | -165.39 | 27.45 | 6.03 | 0.00 |
| Peptone*Peptone | -218.48 | 27.45 | 7.96 | 0.00 |

$R^2$ = 98.4%

**Table 5:** Analysis of variance for ALP production by *Bacillus flexus* FPB17 using CCD criterion

| Source | Sum of Squares | $D_f$ | Mean Square | F Value | p-value |
|---|---|---|---|---|---|
| Model | 3.577E+006 | 9 | 3.975E+005 | 71.95 | 0.00 |
| Ribose | 88066.61 | 1 | 88066.61 | 15.94 | 0.00 |
| Meat Extract | 97577.66 | 1 | 97577.66 | 17.66 | 0.00 |
| Peptone | 1.420E+005 | 1 | 1.420E+005 | 25.70 | 0.00 |
| Ribose*Meat Extract | 58956.63 | 1 | 58956.63 | 10.67 | 0.01 |
| Ribose* Peptone | 34501.70 | 1 | 34501.70 | 6.25 | 0.03 |
| Meat Extract* Peptone | 40602.53 | 1 | 40602.53 | 7.35 | 0.02 |
| Ribose*Ribose | 1.334E+006 | 1 | 1.334E+006 | 241.43 | 0.00 |
| Meat Extract*Meat Extract | 2.006E+005 | 1 | 2.006E+005 | 36.31 | 0.00 |
| Peptone*Peptone | 3.500E+005 | 1 | 3.500E+005 | 63.36 | 0.00 |
| Residual | 55238.88 | 10 | 5523.89 | – | – |
| Lack of Fit | 55235.45 | 5 | 11047.09 | 16103.00 | 0.00 |
| Pure Error | 3.43 | 5 | 0.69 | – | – |

**Optimization of the combination of C and N-sources using CCD**

The medium yielding the highest ALP (ribose 2%, meat extract 0.5% and peptone 0.5%) was further optimized by using CCD as described earlier. Twenty experiments were carried out according to the CCD as shown in Table 3. The experimental ALP yields matched with the predicted yields confirming the soundness of the experimental design based on response surface methodology.

By applying multiple regression analysis on the application data, the following second order polynomial equation was found to explain the ALP production by *Bacillus flexus*:

Y = 1035.40 + 100.42A - 105.71B + 127.51C - 85.85AB + 65.67AC - 71.24BC - 426.47A2 - 165.39 B2 - 218.48C2

Where, Y is the predicted response variable, ALP activity (U/ml) and A, B and C are the values of independent variables ribose, meat extract and peptone respectively.

Regression analysis of this experimental data (Table 4) shows that ribose, meat extract and peptone had positive effect on ALP production ($P < 0.05$). Among N and C-sources, peptone and ribose exhibit highest impact on ALP production as evident by highest linear coefficient followed by the meat extract.

These C and N-sources also showed significant quadratic effect on ALP production. The interaction between ribose-meat extract, ribose-peptone and meat extract-peptone were significant as shown by low P values (<0.05) for interactive terms (Table 5). All the other C-sources including glucose, fructose, sucrose, maltose, mannitol, starch, sodium acetate, cellulose, sorbitol apart from cheap C-sources molasses and whey repressed the *Bacillus flexus* ALP and exhibited the carbon catabolite repression. Analysis of variance for the ALP yields obtained by this design is given in Table 5.

In the present study the value of R (0.984) revealed that the model could explain up to 98.4% variation of ALP production. The P value for the model (0.00) indicated

that the experimental data obtained fitted well with the model and explained the effect of ribose, meat extract and peptone on ALP production by *Bacillus flexus* FPB17. Figures 1, 2 and 3 show the 2D contour plots of ALP production for each pair of C and N source by keeping the other one constant. The optimal combination of the C and N-source of media for ALP production as obtained from the contour plots are as follows: ribose – 2.00 g%; meat extract – 0.68 g% and peptone – 1.00 g%. At these optimum levels of C and N source, ALP production of 1270.21 U/ml was optimized. 3.57-fold increase in the ALP yield was observed after optimization with response surface methodology.

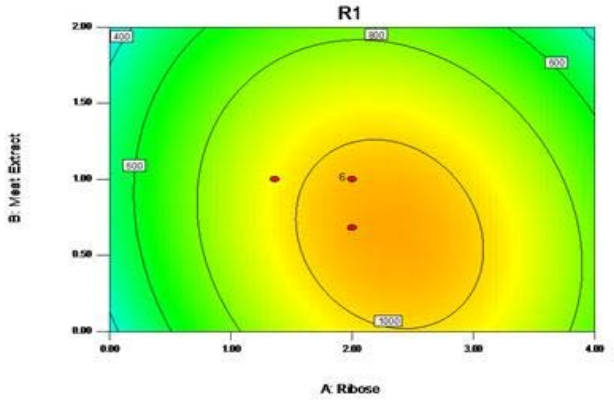


**Figure 1:** Contour plot of ALP activity (U/ml): effect of ribose and meat extract on ALP production

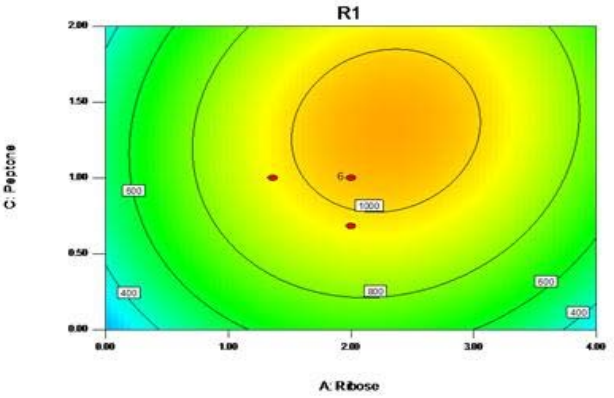


**Figure 2:** Contour plot of ALP activity (U/ml): effect of ribose and peptone on ALP production

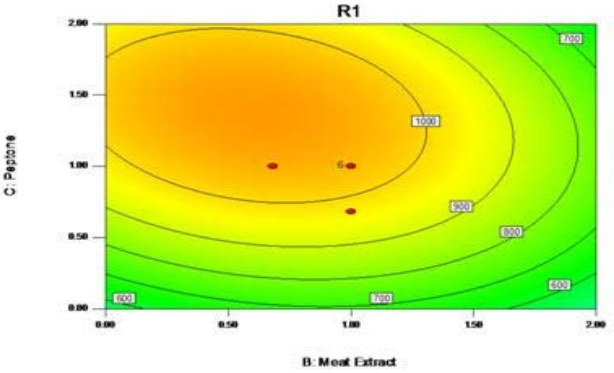


**Figure 3:** Contour plot of ALP activity (U/ml): effect of meat extract and peptone on ALP production

## CONCLUSION

To the best of our knowledge there is no information available in open literature concerning optimization of C and N-sources by applying statistical software for alkaline phosphatase production from polyextremophile *Bacillus flexus*. The obtained results indicate that the C and N-source ribose – 2.00 g%; meat extract – 0.68 g% and peptone – 1.00 g% in production medium have stimulatory effect in alkaline phosphatase production. The C and N-source enhance alkaline phosphatase production by 3.57-fold. The results of this study could be used to design a suitable medium to get higher production of alkaline phosphatase.

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