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Statistical optimization of medium components for improved phytase production by Pseudomonas aeruginosa

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Abstract: Phytases are acid phosphatase enzymes, which efficiently cleave phosphate moieties from phytic acid, thereby generating myo-inositol and inorganic phosphate. Phytase enzyme preparations have a wide range of applications in animal and human nutrition. The addition of phytate-degrading enzyme improves the nutritional value of plant-based foods by enhancing protein digestibility and mineral availability through phytate hydrolysis during digestion in stomach or during food processing. Therefore, the present study was aimed at statistical optimization of phytase production by *Pseudomonas aeruginosa. P. aeruginosa* isolated from rhizospheric soil samples showed phytase activity of 22.165 U/ml. Plackett-Burman statistical experimental design has been employed to optimize culture conditions for maximal enzyme activity by *P. aeruginosa*. Eleven components were screened for their significant effect on phytase production. The main factors that had significant positive effects on phytase production were that sodium phytate, glucose, aeration. Sodium phytate, glucose, aeration, pH and ammonium sulphate were the significant factors, while $MgSO_4.7H_2O$, tween 80, temperature, inoculum size and KNO_3 with $p>0.05$ were considered insignificant. In comparison to the original level 2.3 fold increase in phytase production had been obtained. The enzyme may be a good candidate for use as an environmental-friendly feed additive to enhance the nutritive value of phytate and reduce phosphorus pollution.
Key words: Extracellular phytase, *Pseudomonas aeruginosa*, Plackett-Burman.

Introduction

Phosphorus is an essential constituent of life like nitrogen, but, unlike nitrogen, phosphorus does not have a cycle to constantly replenish its supply. All animal diets must contain adequate amounts of this element. So to meet their phosphorus requirements, inorganic phosphorus like tricalcium phosphate is supplemented in the diet of livestock and poultry animals. This has made phosphorus the third most expensive nutrient in poultry production after energy and protein. At the current extraction and usage rate, the existing phosphate reserves will be exhausted in next 80 years[1].

Phytic acid is the major organic phosphorus source in edible legumes, cereals, oil seeds and nuts. Because of the lack of adequate levels of intrinstic phytases (phytate hydrolyzing enzymes) in

gastrointestinal tract of monogastric animals, phytic acid is excreted in faeces. Further it acts as an anti-nutritional factor by chelating metals such as Ca^{+2}, Mg^{+2}, $Zn^{+}2$ and Fe^{+2} thus making them unavailable, complexing with proteins and thus affecting their digestion and inhibiting enzymes such as a-amylase, trypsin, acid phosphatase and tyrosinase[2]. This unutilized phytate is the origin of phosphorus pollution as it builds up in areas of livestock production leading to eutrophication and algal blooms[3].

In order to overcome this problem, food and feed can be supplemented with phytases for improving phosphorus bioavailability and reducing phosphorus excretion in the areas of intensive live stock populations[4]. Supplementation of animal feeds with phytase will provide swine and poultry producers a safe and effective management tool to reduce nutrient run off by significantly reducing the amount of phosphorus excreted in the manure of the animals[5]. The phytase producing microbes are attracting the attention of scientists all over the world for both environmental and economical reasons. Besides these applications in food and feed industries, phytases have recently been shown to promote the growth of plants[6]. At present, there is no single phytase that is able to meet the diverse needs for all commercial and environmental applications. Therefore, there is an ongoing interest in screening microorganisms, including bacteria for novel and efficient phytases.

The process of fermentation is significantly influenced by various physical and chemical factors. In addition, phytase production is affected by growth conditions, the strain and substrate used for culture, and the availability of nutrients[7]. The conventional methods of optimization are extremely time-consuming and expensive for a large number of variables[8,9]. Statistical plans are currently used to find ways to enhance phytase production at a reduced cost. Optimization of the variables that affect product formation by statistical experimental designs such as Plackett-Burman and response surface methodology eliminates the limitations of 'one variable at a time' approach[10]. There are very few reports on the statistical optimization of phytase production[11]. Therefore the present study was carried out to optimize the medium composition and culture conditions to maximize the production of phytase by *P. aeruginosa* using placket Burmann design.

Materials and Methods

Micro-organism and culture conditions

The organism used throughout this study was locally isolated from rhizospheric soil sample and identified as *P. aeruginosa*. The bacteria was routinely maintained on King's B medium and preserved at 4° C and also maintained at -80°C in peptone water medium containing 20% glycerol.

Growth condition and enzyme production

Basal medium (Phytase screen broth) containing (in g/l): 1.5%glucose, 0.5% $(NH_4)_2SO_4$, 0.05% KCl, 0.01% $MgSO_4.7H_2O$, 0.01% NaCl, 0.01% $CaCl_2.2H_2O$, 0.001% $FeSO_4$, 0.001% $MnSO_4$, pH 7.2 with 0.5% sodium phytate (Sigma) was inoculated with 100µl of 24 hrs bacterial broth (10^8 cfu/ml) and incubated aerobically at 37°C for 5 days. Cells were harvested by centrifugation at 10,000 rpm for 15 min at 4°C and the clear supernatant was used for the enzyme assay.

Enzyme assay

The phytase activity was determined by measuring the amount of liberated inorganic phosphate according to a method of Quan *et al*.[12] (2001). Reaction mixture consisted of 0.8 ml acetate buffer (0.2 M, pH 5.5) containing 1mM sodium phytate and 0.2 ml of cell suspension. After incubation for 30 min at 37°C, the reaction was stopped by adding 1 ml of trichloroacetic acid. One unit of phytase activity was defined as the amount of enzyme required to liberate 1 μmol phosphate per min under assay condition.

Identification of important nutrient components using Plackett–Burman design

Plackett-Burman design, a rapid screening multifactor to find the most significant independent factors,[13,14,15] was used in the present study to screen the important variables that significantly influenced phytase production. In this study, a 12 run Plackett-Burman design (Table 1) with a first-order polynomial equation was applied to evaluate eleven factors (including one dummy variable). Each variable was examined at two levels: -1 for the low level and +1 for the high level. All trials were performed in triplicate and the averages of enzyme activity were treated as the responses. The main effect of each variable was determined with the following equation:

$$E(X_i) = 2(\sum M_i^{+} - M_i^{-}) / N \qquad (1)$$

Where, $E(X_i)$ is the concentration effect of the tested variables. M_i^{+} and M_i^{-} represent phytase activity

from the trials in trials, where the independent variable (X_i) measured was present at high and low concentrations, respectively. *N*, total number of the trials equals to 12. When the sign is positive, the influence of the variable upon phytase production is greater at a high concentration, and when negative the influence of the variable is greater at a low concentration. Using Microsoft Excel, statistical *t*-values for unequal paired samples were calculated for determination of variable significance. Because three trials of the design could not be measured, the main effects of the variables were calculated taking this fact in consideration.

Results and Discussion

The isolate *P. aeruginosa* showed clearance zone by 5 days on phytase screen agar and produced 15.788 U/ml of phytase in liquid medium (phytase screen medium). The production started after 24 hrs of incubation and reached a maximum at 96 hours followed by a parallel decrease in pH. Gaind and Gaur (1991)[16] reported that the drop in solubilization after a maximum value might be attributed to deficiency in nutrients in the
culture medium.

Optimization of culture conditions by Plackett-Burman experimental design (PBD)

In order to enhance phytase production, PBD was employed to identify the key ingredients and the conditions for the best yield of enzyme production. The design screens important variables that affect enzyme production as well as their significant levels but does not consider the interaction effects among the variables. The independent variables examined in Plackett- Burman design and their levels are presented in table 1.

The main effect of each variable upon phytase production was estimated. Analysis of the t- values and p- values of 11 factors showed that sodium phytate, glucose, aeration had positive effects on phytase production, whereas $MgSO_4.7H_2O$, tween 80, temperature, inoculum size, pH, $(NH_4)_2SO_4$ and KNO_3 had negative effects. Sodium phytate, glucose, aeration, pH and ammonium sulphate were the significant factors, these variables had confidence level above 95% in comparison to other variables and thus, were considered to be highly significant for phytase production by *P. aeruginosa*. $MgSO_4.7H_2O$, tween 80, temperature, inoculum size and KNO_3 with $p>0.05$ were considered insignificant. The pareto graph was used to show the effect of all variables on phytase production (Figure1).

Glucose as the secondary carbon source in the tested ranges has the most significant on phytase activity. Bhavsar *et al*. (2010)[17] showed glucose contributed the most to phytase production as observed from the analysis of PBD results. Sunitha *et al.* (1999)[18] discovered that incorporation of glucose to Luria Bertani medium at a level of 2 g/L significantly increased the phytase production by *E. coli* BL21. In our study ammonium sulphate have been identified as important variable, similar results as been reported by Ramachandran *et al.* (2005)[19] who showed that ammonium nitrate as inorganic nitrogen and peptone as organic nitrogen sources stimulated the phytase production in *Rhizopus* spp. and the former was more effective than the latter, whereas Vohra and Satyanarayana[9] identified magnesium sulfate as an important variable for phytase production by the thermophilic mold *Sporotrichum thermophile* by using PBD.

Table 1. The Plackett-Burman design for screening variables in phytase production.

Variable	- level	+ level	Main effect	*t*- value	Significance level
Sodium phytate (%)	0	1.5	5.12	-2.78	**0.0498 (S)**
Glucose	0.1	1	1.57	-12.52	**0.0002 (S)**
$MgSO_4.7H_2O$(%)	0.02	0.1	-0.65	1.34	0.2513 (NS)
Aeration	Static	120 rpm	3.998	-11.57	**0.0003 (S)**
Tween 80 (%)	0.01	0.5	-0.06	-0.183	0.8637 (NS)
Temperature ($^{\circ}$C)	25	37	-0.75	0.5482	0.6127 (NS)
Inoculum size (%)	0.1	1	-0.78	1.7660	0.1521 (NS)
pH	5	7	-0.946	3.75	**0.0199 (S)**
$(NH_4)_2SO_4$ (%)	0.05	0.1	-1.58	-7.74	**0.0015 (S)**
KNO_3 (%)	0.05	0.1	-2.34	2.35	0.0785 (NS)

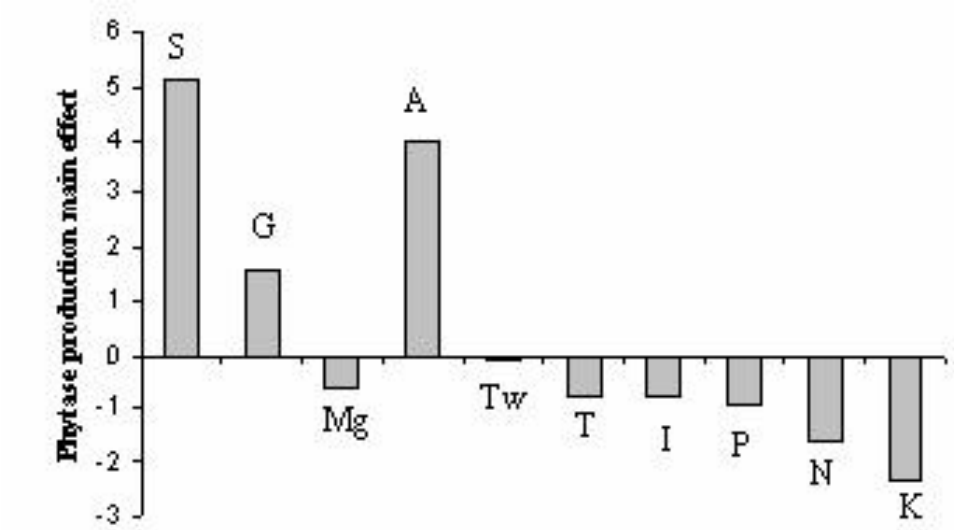

Fig 1. Elucidation of fermentation factors affecting phytase production by *P. aeruginosa*

Phytase activity was significantly influenced by pH, these results are in agreement with that of Andlid *et al.* (2004)[20] who stated that the degradation of phytate during *S. cerevisiae* YS18 cultivation in yeast potato dextrose broth was efficient at initial pH 6.0 and expression of phytase gene was controlled by medium pH. In our study aeration parameter was found to be significant, aeration allows the removal of heat to regulate optimal temperature of growth.

According to the data obtained from the Plackett-Burman experimental results, the medium predicted to be near optimum should be of the following composition (g/L): sodium phytate, 1.5; glucose, 1; $MgSO_4.7H_2O$, 0.02; tween 80, 0.01; $(NH_4)_2SO_4$, 0.05; KNO_3, 0.05; pH,7. Media inoculated with 0.1% (v/v) inoculum of bacterial broth in a total volume of 100ml optimized media and incubated at 37°C for 24 hrs increased phytase production upto 36.456U/ml, which was 2.3 times higher than in the unoptimized medium. By using statistical optimization, a 30% increase in phytase production by *R. pusillus*[11], 85% higher phytase production in *Mucor racemosus*[8] and 74.6% higher phytase production by the yeast *P. anomala*[9] were achieved. These observations clearly suggested that the nutritional and physical requirements of the microbes differs and therefore, need to be optimized for each strain.

Conclusion

P. aeruginosa produces extracellular phytase in simple mineral medium, and placket Burmann design has proved to be effective for optimizing the activity of phytase. Optimization studies resulted in two fold increase of phytases activity. This bacterium is particularly interesting for phytases production with high activity and has potential applications for the reduction of phytate in animal feed.

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