Free and immobilized *Aspergillus oryzae* SBS50 producing protease-resistant and thermostable phytase

Sapna¹ · Bijender Singh¹

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Abstract Optimization for enhanced phytase production by *Aspergillus oryzae* SBS50 in submerged fermentation was investigated using Taguchi design. In first step design, starch, beef extract, magnesium sulphate, ferrous sulphate and Tween 80 were identified as significant factors affecting phytase production. These significant factors were further optimized at four different levels using a second Taguchi design and were observed that 1% starch, 2% beef extract, 3% Tween 80, 0.1% magnesium sulphate and 0.225% ferrous sulphate supported maximum phytase production (47,432 U/L). The use of Taguchi designed experiments resulted in 14.9-fold enhancement in phytase production compared to the medium optimized by ‘one variable at a time’ approach. Furthermore, 4% agar immobilized conidiospores of *A. oryzae* supported high phytase production compared with free cells and other matrices. Agar-immobilized conidiospores resulted in sustained phytase production up to eight repeated batch cycles followed by a decrease in enzyme titres.

Keywords *Aspergillus oryzae* SBS50 · Phytase · Submerged fermentation · Taguchi design · Immobilization

Introduction

Phytic acid (*myo*-inositol hexakisphosphate) is a major form of organic phosphorus (60–90%) in plants where it serve as major storage form of phosphorus and cations

(Vats and Banerjee 2004; Singh and Satyanarayana 2015; Kumar et al. 2017). However, it is not available to monogastric animals (chickens, swine, poultry animals, humans) due to inadequate amount of phytase in their digestive tracts. Since phytic acid cannot be digested, feeds for poultry and piggery are commonly supplemented with inorganic phosphate in diets to meet their daily requirements (Singh and Satyanarayana 2015). The excretion of undigested phytate along with supplementation with inorganic phosphorus imposes global ecological problems (eutrophication) resulting in cyanobacterial blooms, hypoxia and death of marine animals (Vats and Banerjee 2004; Das and Ghosh 2014; Bajaj and Wani 2011, 2015). Phytase hydrolyzes phytic acid to *myo*-inositol and inorganic phosphate via a series of intermediates thus, improving bioavailability of the phosphorus and other minerals (Vats and Banerjee 2004; Singh et al. 2011; Das and Ghosh 2014; Singh and Satyanarayana 2015; Jain et al. 2016; Kumar et al. 2017).

The conventional methods used for optimization are extremely time-consuming and very expensive especially in the optimization of a large number of culture conditions (Vohra and Satyanarayana 2002; Singh and Satyanarayana 2006; Bajaj and Wani 2011, 2015). Sequential statistical designs such as Plackett-Burman and response surface methodology (RSM) have been used widely for screening of media components to enhance phytase production to develop a cost-effective process (Chadha et al. 2004; Singh and Satyanarayana 2006, 2008; Kumari et al. 2016). Taguchi design is an easy and single statistical approach enabling the testing of large number of factors in less number of experiments (Sedghi et al. 2014). This design is not only useful for the identification of significant culture conditions but also can be used to study the interactions among significant factors and to find out their optimal concentrations. However,

✉ Bijender Singh
ohlanbs@gmail.com

¹ Laboratory of Bioprocess Technology, Department of Microbiology, Maharshi Dayanand University, Rohtak, Haryana 124001, India

there is no report on the use of Taguchi design for phytase production so far. Taguchi design has orthogonal arrays (OAs) that can reduce the number of experiments when compared with other factorial designs (Sedghi et al. 2014).

In the last few years, scientists have been more focused on the use of immobilized whole cells and biocatalysts because it is a very useful technique that permits efficient reuse of cells (Hemachander et al. 2001; Singh and Satyanarayana 2008). Whole cell immobilization is advantageous over immobilized enzymes because it eliminates the need for enzyme purification and extraction steps, yields higher enzyme titres and higher operational stability (Bucke 1987). Immobilization involving cell entrapments in different polysaccharides has been the most successful approach that permits the retention of cell viability and activity, making the nutrients available for growth and leading to high cell densities (Bucke 1987). Agar, agarose, collagen, cellulose, chitosan and alginate have been employed for microbial immobilizations (Park and Chang 2000). Alginate being food grade polymer has been used for immobilization of different types of cells such as RBCs, mitochondria, animal cells, protoplasts and chloroplasts (Park and Chang 2000). Alginate has been successfully utilized for immobilization of conidiospores of *S. thermophile* (Singh and Satyanarayana 2008) and cells of *Candida krusei* for phytase production (Quan et al. 2003). *Aspergillus oryzae* SBS50 secreted an extracellular, thermostable and protease-resistant phytase in submerged (Sapna and Singh 2013) and solid-state (Sapna and Singh 2014, 2015) fermentations. Therefore, this investigation was planned to optimize the conditions for phytase production by *A. oryzae* SBS50 in submerged fermentation and also to study the effect of immobilizing conidiospores on phytase production.

Materials and methods

Microorganism and culture conditions

Aspergillus oryzae SBS50 was isolated from a soil sample collected from Rohtak, Haryana (India) and was routinely grown on potato dextrose agar (PDA) medium (Sapna and Singh 2013). Spore suspension of the fungus was prepared from 3 day old PDA slants by adding 30 ml of normal saline containing Tween 80 (0.1% v/v) to obtain 4×10^7 spores/ml and used as inoculum for submerged fermentation. The cultures were maintained on PDA slants at 4 °C and in glycerol at −20 °C.

Phytase production

The Erlenmeyer flasks containing 50 ml medium (as per experimental design) were inoculated with 1 ml of spore

suspension having a viable spore count of 4×10^7 (CFU/ml) prepared from 3 days old culture of *A. oryzae*. The flasks were incubated at 35 °C and 200 rpm in an incubator shaker. At desired intervals, cultures were obtained by filtration and cell-free culture filtrates were used in phytase assays.

Phytase assay

Phytase was assayed by determining the amount of phosphate liberated using calcium phytate as the substrate at 50 °C and pH 5.0 (Sapna and Singh 2013, 2014, 2015, Fiske and Subbarow 1925). One unit of phytase is defined as the amount of enzyme required to liberate 1 μmol of inorganic phosphate per min under the assay conditions using KH_2PO_4 as the standard.

Identification and optimization of medium components for phytase production

Taguchi design methodology is an experimental strategy in which the effects of multiple factors are studied simultaneously by running test at various levels of the factors. A two step optimization design was adopted for the identification of significant factors during the first step followed by their optimization in the second step. Eleven different fermentation parameters such as temperature, pH, incubation period, starch, beef extract, ammonium nitrate, Tween 80, dipotassium hydrogen phosphate, ferrous sulphate, potassium chloride and magnesium sulphate were studied using the first Taguchi design of experiments. The range of these parameters was selected on the basis of our preliminary studies for phytase optimization using this isolate 'one variable at a time'. Each variable was represented at two values, high and low denoted by (+) and (−), respectively (Table 1). The important variables selected during the first stage were further optimized using same design keeping each parameter at four levels. To validate the optimized methodology, fermentation experiments were performed in triplicates and the samples were collected for enzyme assays.

Scaling up of phytase production in flasks

The validation of model and feasibility of phytase production was tested in flasks (250–1000 ml) with varied volume of the medium (50–200 ml) and the medium containing (%) starch 1.0, beef extract 2.0, Tween 80 3.0, magnesium sulphate 0.1 and ferrous sulphate 0.225. The flasks were sterilized at 121 °C for 15 min and cooled media was inoculated with *A. oryzae* spore suspension and incubated at 35 °C and 200 rpm for 72 h. Culture filtrates were withdrawn aseptically at desired intervals for the estimation of phytase activity.

Table 1 Eleven different cultural factors screened using first Taguchi design

S. No.	Factor	Low value	High value
1	Temperature (°C)	30	50
2	Incubation period (day)	3.0	5.0
3	Starch (%)	1.0	3.0
4	Beef extract (%)	0.5	3.0
5	Ammonium nitrate (%)	0.5	2.0
6	Tween 80 (%)	1.0	5.0
7	Dipotassium hydrogen phosphate (%)	0.0	0.05
8	Ferrous sulphate (%)	0.0	0.1
9	Potassium chloride (%)	0.0	0.1
10	Magnesium sulphate (%)	0.0	0.1
11	pH	5.0	7.0

Immobilization of fungal conidiospores for phytase production

The spore suspension of *A. oryzae* was prepared in normal saline containing 0.1% (v/v) Tween-80, washed and mixed with sterile sodium-alginate (4%). The mixture was extruded through a sterile syringe needle into a chilled sterile solution of 0.2 M CaCl₂ aseptically with constant stirring. The beads were cured in the same solution for 1 h, washed with sterile double distilled water and stored at 4 °C until use (Singh and Satyanarayana 2008). In preparing other matrices, 4% agar-agar and 1% of agarose was mixed well with spore suspension and poured into sterile petriplates, allowed to harden and cut into blocks (5 × 5 × 3 mm³) (Singh and Satyanarayana 2008). A known number of calcium-alginate beads and blocks of other matrices (with equal number of CFU) were used for inoculating 50 ml medium in 250 ml Erlenmeyer flasks. Phytase production of immobilized agar conidiospores was also studied in repeated batch fermentation by transferring agar blocks into fresh medium after 72 h. Phytase production by agar-immobilized conidiospores was monitored for 8 cycles during batch fermentation in medium optimized by ‘one variable at a time’ approach and Taguchi design.

All experiments were carried out in triplicates and their mean values are presented along with the standard error.

Results and discussion

Identification and optimization of medium components for phytase production

Aspergillus oryzae SBS50 secreted a protease-resistant and acid stable phytase at pH 5.0, 35 °C and 200 rpm after 96 h with starch and beef extract in submerged fermentation (Sapna and Singh 2013). Therefore, the present investigation

was planned to further optimize phytase production in SmF using Taguchi designs. Taguchi design is used for screening various culture conditions and to study their interactions during microbial metabolite production (Assemi et al. 2012). The design is used to screen important culture conditions that effect metabolite production and also to study interactions among these selected parameters. Eleven selected culture parameters were screened using first Taguchi design (Table 2) and it was observed that starch, beef extract, magnesium sulphate, Tween 80 and ferrous sulphate significantly affected the phytase production (Fig. 1). Remaining variables had limited contribution towards phytase production and, therefore, they were considered as less significant variables. The influence of these five selected variables on the phytase production by *A. oryzae* SBS50 was tested at four different levels (Table 3) in second Taguchi experimental design (Table 4). Highest phytase production (47432 U/L) was detected in experiment number 12. Table 5 presents the effect of each variable with its *P* value, *F* value, contribution (%) and degree of freedom. Beef extract and magnesium sulphate showed high contribution for phytase production. The optimum values of selected variables are as Tween 80 3.0%, beef extract 2.0%, magnesium sulphate 0.1%, ferrous sulphate 0.225% and starch 1.0%. Phytase production was increased as the concentration of Tween 80 increased up to 3% level, after that enzyme production declined. Interaction of Tween 80 and beef extract suggested that optimum phytase production was recorded at 3% level of Tween 80 and 1% starch followed by a decline afterwards. The maximum experimental response for phytase production was 47,432 U/L. A two step Taguchi experimental design resulted in 14.9-fold enhancement in phytase production as compared to the medium optimized by ‘one variable at a time’ (3170.37 U/L) approach. Assemi et al. (2012) used the Taguchi method to evaluate the effects of all 16 feed ingredients of a diet on some biological characteristics of *Helicoverpa armigera*. Taguchi methodology has been successfully used for the optimisation of 1, 3-propanediol

Table 2 Experimental designs and results for 11 selected factors used in first Taguchi design

Run No.	Temperature (°C)	Incubation period (d)	Starch (%)	Beef extract (%)	Ammonium nitrate (%)	Tween 80 (%)	Dipotassium phosphate (%)	Ferrous sulphate (%)	Potassium chloride (%)	Magnesium sulphate (%)	pH	Phytase production (U/L $\pm$ S.D.)
1	30	5.0	3.0	0.5	2.0	5.0	0.0	0.1	0.0	0.1	5.0	35671.5 $\pm$ 1783.5
2	40	3.0	1.0	3.0	2.0	5.0	0.0	0.1	0.1	0.0	5.0	20138.2 $\pm$ 1006.9
3	30	3.0	3.0	3.0	2.0	1.0	0.0	0.0	0.1	0.1	7.0	3342.71 $\pm$ 167.1
4	40	3.0	3.0	3.0	0.5	1.0	0.05	0.1	0.0	0.1	5.0	6258.4 $\pm$ 326.4
5	30	3.0	1.0	0.5	0.5	5.0	0.05	0.1	0.1	0.1	7.0	31805.7 $\pm$ 1590.2
6	40	5.0	1.0	3.0	0.5	5.0	0.0	0.0	0.0	0.1	7.0	25634.3 $\pm$ 1281.7
7	30	5.0	3.0	3.0	0.5	5.0	0.05	0.0	0.1	0.0	5.0	22304.1 $\pm$ 1115.2
8	30	3.0	1.0	0.5	0.5	1.0	0.0	0.0	0.0	0.0	5.0	4539.79 $\pm$ 226.9
9	40	3.0	3.0	0.5	2.0	5.0	0.05	0.0	0.0	0.0	7.0	31922.1 $\pm$ 1596.1
10	40	5.0	1.0	0.5	2.0	1.0	0.05	0.0	0.1	0.1	5.0	2066.51 $\pm$ 103.3
11	30	5.0	1.0	3.0	2.0	1.0	0.05	0.1	0.0	0.0	7.0	3440.52 $\pm$ 172.1
12	40	5.0	3.0	0.5	0.5	1.0	0.0	0.1	0.1	0.0	7.0	4837.82 $\pm$ 241.8

Fig. 1 Pareto graph showing the effect of different variables on phytase production by *Aspergillus oryzae* SBS50

production by *K. pneumoniae* 141B (Jalasutram and Jetty 2011). Lipase production in *Bacillus* F3 was successfully optimized by Taguchi method (Heravi et al. 2008). However, other statistical techniques, such as Plackett-Burman and response surface methodology have been widely used for enhanced phytase production by *A. japonicus* (Maller et al. 2014), *A. ficuum* NRRL3135 (Coban and Demisa 2014), *Nocardia* sp. MB36 (Bajaj and Wani 2011), *A. niger* NCIM563 (Bhavsar et al. 2012) and *S. thermophile* (Singh and Satyanarayana 2006, 2008; Kumari et al. 2016).

Scaling up of phytase production in flasks

The model was validated by repeating the experiment under optimized conditions that resulted in 47557 U/L of phytase production by *A. oryzae*. Phytase production was sustainable (45967 ± 2298.3 to 47557 ± 2377.8 U/L) in Erlenmeyer flasks containing varied volumes of optimized medium. There was a slight decline in phytase production in 1 L flasks, which could be due to the reduction in dissolved oxygen and inadequate mixing with increased volume (Vohra and

Table 3 Ranges of the significant factors used for optimization using second Taguchi design

S. No.	Selected factor	Range of factors			
		1	2	3	4
1	Starch (%)	1	2	3	4
2	Beef extract (%)	0.5	1.0	1.5	2.0
3	Tween 80 (%)	1	2	3	4
4	Magnesium sulphate (%)	0.05	0.10	0.15	0.20
5	Ferrous sulphate (%)	0.075	0.150	0.225	0.300

Table 4 Experimental design and results of second Taguchi design used for phytase production

Run No.	Tween 80 (%)	Beef extract (%)	Magnesium sulphate (%)	Starch (%)	Ferrous sulphate (%)	Phytase production (U/L $\pm$ S.D.)
1	4.0	1.5	0.1	4.0	0.075	32108 $\pm$ 1605.8
2	4.0	0.5	0.2	2.0	0.225	23398 $\pm$ 1169.2
3	1.0	2.0	0.2	4.0	0.300	7935 $\pm$ 396.4
4	2.0	1.5	0.2	1.0	0.150	22979 $\pm$ 1148.1
5	4.0	1.0	0.15	1.0	0.300	13850 $\pm$ 692.7
6	1.0	0.5	0.05	1.0	0.075	12220 $\pm$ 611.2
7	2.0	0.5	0.1	3.0	0.300	2788 $\pm$ 138.4
8	1.0	1.5	0.15	3.0	0.225	9169 $\pm$ 458.8
9	2.0	2.0	0.15	2.0	0.075	17297 $\pm$ 864.9
10	1.0	1.0	0.1	2.0	0.150	18927 $\pm$ 946.2
11	4.0	2.0	0.05	3.0	0.150	42448 $\pm$ 2122.6
12	3.0	2.0	0.1	1.0	0.225	47432 $\pm$ 2371.4
13	3.0	0.5	0.15	4.0	0.150	31828 $\pm$ 1591.3
14	3.0	1.5	0.05	2.0	0.300	39746 $\pm$ 1987.9
15	3.0	1.0	0.2	3.0	0.075	21302 $\pm$ 1065.1
16	2.0	1.0	0.05	4.0	0.225	26193 $\pm$ 1309.1

Table 5 Analysis of variance showing various parameters and their interaction affecting phytase production by *A. oryzae*

Term intercept	DF	Sum of squares	Mean sq require	% Contribution
Tween 80	3	4.888E+0.007	1.629E+0.007	4.47
Beef extract	3	3.605E+0.008	1.202E+0.008	33.00
Magnesium sulphate	3	3.630E+0.008	1.210E+0.008	33.23
Starch	3	1.148E+0.008	3.825E+0.007	10.50
Ferrous sulphate	3	2.053E+0.008	6.842E+0.007	18.78

Satyanarayana 2002; Singh and Satyanarayana 2006, 2008; Kumar and Satyanarayana 2007). Sustainable phytase production was also reported in *A. ficuum* NRRL3135 (Coban and Demisa 2014), *P. anomala* (Vohra and Satyanarayana 2002) and *S. thermophile* (Singh and Satyanarayana 2006, 2008).

Immobilization of conidiospores for phytase production

Now, scientists are more focused on the use of immobilized cells and biocatalysts because it is a very useful technique that

allows efficient reuse of cells and biocatalysts (Hemachander et al. 2001). Whole cell immobilization eliminates the need of enzyme purification, its extraction, higher enzyme activity, higher operational stability, provides resistance to environment perturbations and lower cost (Hemachander et al. 2001; Singh and Satyanarayana 2008; Kaur and Satyanarayana 2010). Phytase production by fungal conidiospores entrapped in different gels was studied. Agar entrapped fungal conidiospores secreted higher enzyme titers (3192.70 ± 18.9 U/L) than those entrapped in alginate (2292.28 ± 16.2 U/L) and agarose (2432.15 ± 11.7 U/L). Fungal growth around beads/

Fig. 2 Photographs of **a** immobilized agar blocks and **b** immobilized sodium alginate beads showing fungal growth noticeable with naked eye

gels was noticeable with the naked eye (Fig. 2) and rectangle shaped agar blocks become rounded after fungal growth during batch fermentation. Agar blocks have not been used earlier for immobilization of fungal spores for phytase production. Starch agar beads have been utilized successfully for immobilization of *E. coli* for phytase production (Kuek 1991). Conidiospores of *S. thermophile* (Singh and Satyanarayana 2008) and *P. purpurogenu* GE1 (Awad et al. 2015) immobilized in alginate and carrageenan were used for phytase production, respectively. Ca-alginate immobilized *C. krusei* cells (Quan et al. 2003) and sporangiospores of *T. indiciae-seudaticae* (Kumar and Satyanarayana 2007) have been used successfully for phytase and glucoamylase production, respectively. Alginate entrapped cells of *P. anomala* were used for phytase production (Kaur and Satyanarayana 2010). Phytase production by immobilized fungus was studied in repeated batch fermentations. The diameter of the agar blocks increased gradually with every cycle and the mycelial growth outside the beads was noticeable with the naked eye. Phytase production by immobilized *A. oryzae* was sustainable up to eight cycles with agar blocks under conditions optimized by 'one variable at a time' approach (Fig. 3a) and Taguchi design (Fig. 3b) with a marked increase in second cycle. The heavy mycelial growth around the bead might have increased resistance to nutrients and oxygen transfer that declined enzyme titres. Similarly, phytase production by *S. thermophile* immobilized spores was sustainable over five successive batches that declined afterwards (Singh and Satyanarayana 2008). Immobilization of permeabilized *P. anomala* cells in alginate showed reusability with sustained phytase activity (Kaur and Satyanarayana 2010). *Aspergillus phoenicis* produced sustainable glucoamylase over five sequential batches (Awad et al. 2015), while *T. indiciae-*

Fig. 3 a Repeated batch fermentation by agar immobilized conidiospores in the medium optimized by 'one variable at a time' approach. **b** Repeated batch fermentation by agar immobilized conidiospores in the medium optimized by Taguchi design

seudaticae secreted sustainable glucoamylase titres up to eight repeated batch fermentations (Kumar and Satyanarayana 2007).

Conclusions

Aspergillus oryzae SBS50 secreted high phytase titer at 35 °C, pH 5.0 after 96 h in the medium containing starch, beef extract and Tween 80. Starch, beef extract, magnesium sulphate, ferrous sulphate and Tween 80 were identified as important factors affecting phytase production using the Taguchi design. Statistical optimization resulted in 14.9-fold improvement in phytase production. Agar-agar (4%) was found as the best matrix for immobilization of *A. oryzae* conidiospore for phytase production that secreted sustainable phytase up to eight repeated batch fermentations under optimized conditions.

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Compliance with ethical standards

Conflict of interest All the authors declare that they have no conflict of interest.

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