JOURNAL OF PURE AND APPLIED MICROBIOLOGY, May 2015. Vol. **9**(Spl. Edn. 1), p. 257-263

Variables affecting the Recombinant Phytase Production from *Escherichia coli* DH5α

Rafidah Mohd Ariff[1], MohdYazidAbd. Manap[1], Aini Ideris[2], Azhar Kassim[3], Abd-El Aziem Farouk[4] and Anis Shobirin Meor Hussin[1]*

[1]Department of Food Technology, Faculty of Food Science and Technology, Universiti Putra Malaysia, Serdang, Selangor, Malaysia.
[2]Department of Science Clinical Study, Faculty of Veterinary Science, Universiti Putra Malaysia, Serdang, Selangor, Malaysia
[3]Department of Animal Science, Faculty of Agriculture, Universiti Putra Malaysia, Serdang, Selangor, Malaysia.
[4]Department of Biotechnology, Faculty of Science, Taif University, P. Box 888, AlHawiya, Kingdom of Saudi Arabia.

(Received: 20 November 2014; accepted: 07 January 2015)

Screening for the important variables is part of the optimization study of enzyme production. It is important indetermining the influential variables that give a significant effect on the enzyme production. Thus, the objective of this study was to determine variables that are effecting the recombinant phytase production from *Escherichia coli* DH5±. Through the application of the Fractional Factorial Design, the variables of seed age, inoculum level, time of induction, L-arabinose concentration and post-induction time were highly significant for the recombinant phytase production as their *p*-values were less than 0.05.Thus, minimal changes of this factor mat effect recombinant phytase production from *E. coli* DH5±.

Key words: Screening; Variables, Optimization,Recombinant Phytase, Fractional Factorial.

Phytase [myo-inositol (1, 2, 3, 4, 5, 6) hexaphosphate phosphohydrolase] is able to hydrolyze the phytate molecules in a sequential and stepwise manner of plant-based foods such as cereals and legumes, subsequently releasing the bound phosphorus and other important minerals such as calcium, magnesium and protein that are attached to the phytate, hence improving nutrient utilization of monogastric animals such as fish, pigs, birds and humans (Greiner and Konietzny, 2006; Lei and Porres, 2003). However, each strain of microorganism demands its own optimum conditions in order to produce a high level of enzyme production (Schumann and Ferreira, 2004). Therefore, optimization of various parameters involved during the fermentation process is needed to achieve the desired goal (Ries and Macedo, 2011; Zouari*et al.*, 2010). Moreover, the type of strain use, substrate, growth conditions and nutrients may affect the production of phytase (Vats and Banerjee, 2004). In addition, optimization involves a sequential step, where the screening for the significant factors, referred to as a primary step, prior to optimization in order to determine the optimum levels of each significant factor (Li *et al.*, 2008; Kaur and Satyanarayana, 2005).

Screening for the significant factors might be achieved by using statistical methods such as

* To whom all correspondence should be addressed.
Tel: +60389468347; Fax: +603-89423552;
E-mail : shobirin@upm.edu.my

factorial design and Plackett-Burman design (Chakraborty *et al.*, 2009). Various studies reported that factors such as medium components used during fermentation and cultivation conditions have an effect on the phytase production. For example, by using the statistical design, the factors of period of incubation, glucose, magnesium sulfate, ammonium sulphate, peptone, Tween 80, starch and sodium phytate appeared to be most significant for phytase production by *Sporotrichum thermophile* (Singh and Satyanarayana, 2008a; Singh and Satyanarayana, 2008b; Singh and Satyanarayana, 2006).

Meanwhile, initial pH, oats and ammonium sulfate were significant in their effect on phytase production by *Kodamaeaohmeri* BG3 (Li *et al.*, 2008), urea, cane molasses and inoculum density were significant for phytase production in *P. anomala* (Kaur and Satyanarayana, 2005), while ammonium salts $(NH_4)_2SO_4$) and starch were significant for phytase production by *A. ficuum* NRRL3135 (Bogar*et al.*, 2003a). In addition to the $(NH_4)_2SO_4$), casein and glucose were significant factor for phytase production in *Mucor racemosus* (Bogar *et al.*, 2003b).

MATERIALS AND METHODS

Chemicals

Phytic acid as a dodecasodium salt was purchased from Sigma Chemical Co. (St. Louis, MO, USA). All other chemicals were of analytical grade.

Bacterial culture

An 80 percent (%) glycerol stock of *Escherichia coli* DH5α that was previously transformed with an ES-TOPO plasmid carrying the phytase gene from *Enterobacter sakazakii* ASUIA279 was provided by the Department of Biotechnology Engineering, International Islamic University Malaysia (IIUM) and stored at -80 degree celsius (°C). This *E. coli* DH5α culture was used as the expression host for phytase production (U/ml). This intracellular phytase enzyme was expressed by L-arabinose induction under the regulation of the araBAD promoter (PBAD).

Fermentation condition for recombinant phytase production from Escherichia coli DH5α

Recombinant *E. coli* DH5α cells were incubated for 24 hours (h) at 37 °C on Luria Bertani (LB) agar plate supplemented with 100 microgram/milliliter (µg/ml) ampicillin, by using a Memmert incubator (Schwabach, Germany). After 24 hours, a single colony was inoculated into LB broth supplemented with 100 µg/ml ampicillin in an Erlenmeyer flask and was aerobically grown in the incubator shaker (model SI-600, JEIO TECH, Seoul, Korea) at 37 °C and agitated at 200 rotations per minute (rpm) for 18 hours. Later, 10 % volume/volume (v/v) of the culture was sub-cultured into new LB broth, supplemented with 100 µg/ml ampicillin, in an Erlenmeyer flask and grown also at 37 °C and agitated speed of 200 rpm for the 3 to 11 h seed age preparation. About 2.5 to 7.5 % (v/v) (equivalent to $3.2 \times 10^8 – 1.7 \times 10^9$ Colony Forming Unit (CFU/ml cells)] of this culture was used as inoculum for 100 ml fermentation medium (Nuge, 2011), grown in a 500 ml Erlenmeyer flask supplemented with 100 µg/ml ampicillin. Then, when the cell concentration of the growing bacterial culture reached ODs of 0.3, 0.5 and 0.7 equivalent to 3.8×10^8 - 1.0×10^9CFU/ml cells, after measuring at 600 nanometer (nm) using spectronic GENESYS 20 visible spectrophotometer (Thermo Scientific, Swedesboro, NJ 08085 U.S.A.), the induction was done with different levels of L-arabinose concentrations: 0.002, 1 and 2 %,. Finally, the cultures were harvested in between 2.5 and 17.5 h after induction.

Recombinant phytase extraction from Escherichia coli DH5α

The bacterial cells were harvested by centrifugation at 13,751 G-Force (g) for 20 minutes (min) at 4 °C using a Sigma 3-18K centrifuge (Sartorius Stedim, Göttingen, Germany). The bacterial pellet was collected and dissolved in 100 millimolar (mM) sodium acetate buffer, pH 5, and the cells were disrupted using a 150 V/T ultrasonic homogenizer (Biologics Inc., Manassas, Virginia, USA) equipped with a stepped titanium microtip, 5/32" (3.9 mm) in diameter and 255.8 mm in length (Model 150VT). The cells were disrupted for 30 seconds (sec) with 30 sec cooling periods by using 30 Watt acoustic power and a 50 % duty cycle at 20 kilohertz (kHz)for 1 min. The samples were kept in salt ice bath during the ultrasonication process to prevent overheating, subsequently preventing the proteins from denaturing (Ho *et al.*, 2006). Then, the sonicated cells were centrifuged at 12,581 g, 4 °C, for 30 min to remove the cell debris by using a

Sigma 3-18K centrifuge (Sartorius Stedim, Göttingen, Germany). The supernatant was analyzed for phytase activity (U/ml). The appropriate processing volume for this probe ranged from 300 µl to 15 milliliter (ml).

Measurement of phytase activity (U/ml)

Phytase activity was determined using a modified method as described by Meor Hussin *et al.*(2010). The assay mixture consisted of 299 microliter (µl) of 100 mM sodium acetate buffer (pH 5) and 100 µl of 3.6 mM sodium phytate. The assay mixture was pre-incubated at 50 °C for 5 min using a shaking water bath (PROTECH®, Selangor, Malaysia). Furthermore, the enzymatic reaction was started by adding 1 µl of diluted enzyme solution. The assay mixture was further incubated at 50 °C for 30 min. Then, the inorganic liberated phosphate was measured according to an ammonium molybdate method (Heinonen and Lahti, 1981) with some modifications. A 1.5 ml of a freshly prepared stop solution consisting of acetone: 5 N H_2SO_4: 10 mM ammonium molybdate (2:1:1 v/v), was added to the assay mixture before adding 100 µl of 1 molar (M) citric acid. Any cloudiness was removed by centrifugation prior to measure the absorbance at 355 nm. In order to calculate the enzyme activity, a calibration curve was produced over the range of 5-600 mmol phosphate ($\mu = 8.7\ cm^2/nmol$). Activity (units) was expressed as 1 micromol (µmol) inorganic phosphate liberated in 1 min under assay conditions (Lee *et al.*, 2007; Quan *et al.*, 2002). Stop solution was added to the assay mixture prior to adding the enzyme for the blank run. The reaction was performed in triplicate.

RESULTS AND DISCUSSION

Experimental conditions

The matrix of fractional factorial design used during the screening study is shown in Table 4.1. The five central points have been added to the experimental design to determine the pure error sum-of-square and to determine the repeatability of the experiment and the experiment was randomly

Table 1. Fractional factorial design showing experimental and predicted phytase activity (U/ml)

Run Order	Seed age (h), x_1	Inoculum level (%, v/v), x_2	L-arabinose conc. (%, w/v), x_3	Cell conc. at 600nm, x_4	Harvesting time (h),x_5	Phytase activity (U/ml)	
						Experimental	Predicted
1*	7(0)	5(0)	1.001(0)	0.5(0)	10(0)	2420.56	2518.10
2	11(+1)	2.5(-1)	0.002(0)	0.3(-1)	2.5(-1)	1276.67	1280.34
3*	7(0)	5(0)	1.001(0)	0.5(0)	10(0)	2456.31	2518.10
4	11(+1)	2.5(-1)	2(+1)	0.7(+1)	2.5(-1)	541.31	699.45
5	11(+1)	2.5(-1)	0.002(-1)	0.7(+1)	17.5(+1)	1613.71	1611.63
6	3(-1)	7.5(+1)	0.002(-1)	0.7(+1)	17.5(+1)	4945.81	4787.66
7	11(+1)	2.5(-1)	2(+1)	0.3(-1)	17.5(+1)	896.22	736.48
8	3(-1)	7.5(-1)	2(+1)	0.3(-1)	17.5(+1)	1240.92	1237.25
9	11(+1)	7.5(+1)	0.002(-1)	0.3(-1)	17.5(+1)	2188.21	2190.28
10	3(-1)	2.5(-1)	0.002(-1)	0.3(-1)	17.5(+1)	4230.87	4389.02
11	3(-1)	2.5(-1)	2(+1)	0.7(+1)	17.5(+1)	980.48	984.15
12	3(-1)	7.5(+1)	0.002(-1)	0.3(-1)	2.5(-1)	454.49	614.24
13	3(-1)	2.5(-1)	0.002(-1)	0.7(+1)	2.5(-1)	2688.66	2528.92
14*	7(0)	5(0)	1.001(0)	0.5(0)	10(0)	2576.31	2518.10
15	3(-1)	7.5(+1)	2(+1)	0.7(+1)	2.5(-1)	1608.60	1610.67
16	11(+1)	7.5(+1)	2(+1)	0.7(+1)	17.5(+1)	3431.68	3591.42
17	11(+1)	7.5(+1)	2(+1)	0.3(-1)	2.5(-1)	1399.23	1241.08
18	3(-1)	2.5(-1)	2(+1)	0.3(-1)	2.5(-1)	1251.13	1249.06
19*	7(0)	5(0)	1.001(0)	0.5(0)	10(0)	2277.57	2518.10
20*	7(0)	5(0)	1.001(0)	0.5(0)	10(0)	2859.73	2518.10
21	11(+1)	7.5(+1)	0.002(-1)	0.7(+1)	2.5(-1)	1971.17	1967.50

*Central points

run. The variables and levels have been chosen based on the study done by Pan *et al.* (2008) and Sunitha *et al.* (2000) with modifications made.

Data analysis

Table 1shows the experimental and predicted values of phytase activity (U/ml) for fractional factorial design. The estimated effects, estimated coefficient, *T*-value and the *p*-value of the five variables are listed in Table 2. Based on the results, it shows that variables x_1, x_2, x_3, x_4 and x_5 significantly contributed to the phytase production as their *p*-values were less than 0.05 which is related to the high effect values. In addition, Table 3 shows the percentage of contribution (%) of each variable and their interactions for phytase production. The % was calculated as follows;

$$\% \text{ of contribution: } \frac{\text{Absolute effect values of each variables or interaction}}{\text{Total effect values}} \times 100$$

Total effect values

Based on the results, variable x_5 and x_3 contributed bigger effects on the phytase production compared to the other variables. The % of contribution was proportionally related to the coefficient values. Regardless of the positive (+) or negative (-) sign, the highest coefficient values referred to the most significant variables (**Table 2**). Based on the % of contribution or coefficient values, the order of those variables starting from the highest contribution to the lowest is as follows;

$x_5 > x_3 > x_4 > x_1 > x_2$

The (+) and (-) signs of the coefficient values refer to the positive and negative effects of the variables to the response. The positive and negative effects specified the relationship between the variables and the response. Variables x_1 and x_3 showed (-) sign of their coefficient values which indicates that these variables negatively affect the phytase production. Meanwhile, the other three variables, x_2, x_4 and x_5,showed (+) sign of their coefficient values which means that these variables

Table 2. Estimated effects and coefficient for the phytase activity (U/ml)

Term	Effect	Coefficient	Std. error coefficient	*t*-value	*p*-value
Constant		1919.9	59.27	32.39	0.000
x_1	-510.3	-255.2	59.27	-4.31	0.004*
x_2	470.1	235.1	59.27	3.97	0.005*
x_3	-1002.5	-501.3	59.27	-8.46	0.000*
x_4	605.5	302.7	59.27	5.11	0.001*
x_5	1042.1	521.0	59.27	8.79	0.000*
x_1x_2	695.5	347.7	59.27	5.87	0.001
x_1x_3	807.2	403.6	59.27	6.81	0.000
x_1x_5	-306.7	-153.4	59.27	-2.59	0.036
x_2x_3	532.7	266.3	59.27	4.49	0.003
x_2x_4	1063.1	531.6	59.27	8.97	0.000
x_2x_5	551.2	275.6	59.27	4.65	0.002
x_3x_5	-604.8	-302.4	59.27	-5.1	0.001
Ct Pt		598.2	121.47	4.92	0.002

*p-value<0.05

Table 3. Percentage of contribution (%) of the significant terms for phytaseproduction

Variable	Effect	Percentage of contribution (%)
x_1	-510.3	6.23
x_2	470.1	5.74
x_3	-1002.5	12.24
x_4	605.5	7.39
x_5	1042.1	12.72
x_1x_2	695.5	8.49
x_1x_3	807.2	9.85
x_1x_5	306.7	3.74
x_2x_3	532.7	6.50
x_2x_4	1063.1	12.98
x_2x_5	551.2	6.73
x_3x_5	604.8	7.38
Total	8191.7	

Table 4. Analysis of variance (ANOVA) for the phytase activity (U/ml)

Source	DF	Sum of square	Mean square	*F*-value	*p*-value
Main Effects	5	11756002	2351200	41.83	0.000
2-Way interaction	7	13251752	1893107	33.68	0.000
Curvature	1	1362985	1362985	24.25	0.002
Residual Error	7	393472	56210		
Lack of Fit	3	202185	67395	1.41	**0.363***
Pure Error	4	191288	47822		
Total	20				

Coefficient of determination, R^2 = 0.985, R^2 (adjusted) = 0.958
DF = degree of freedom, ***p*>0.05

positively affect the phytase production.

In a linear model, the phytase production is modeled as a function of significant main and interaction terms ($p<0.05$). Meanwhile, the goodness-of-fit of the specified linear model is measured by a coefficient of determination, R^2. In the linear model of phytase production, a relatively higher value of R^2 (0.958) was obtained (Table 4). As it is mentioned in the Table 4, the linear model of phytase production produced a *p*-value of 0.363 for the lack-of-fit (LOF), which is higher than any reasonable significant level. Thus, a high value of R^2 and the non-significant value of lack of fit implied that the experimental data fits to the model equation and the model is significant. The fractional factorial design is one of the statistical methods that have been broadly used in screening study, aimed to select the significant variables for the response. For example, fractional factorial design has been applied to determine the most significant variables involved in the production of xylitol (Carla and Roberto, 1999), in the production of spore by *Verticillium lecanii* (Shi *et al*., 2009) and in the production of recombinant xynalase from *E. coli* DH5α (Farliahati *et al.*, 2010). Other than using fractional factorial design, Plackett-Burman design was also used for screening study (Pelinski *et al.*, 2012; Nelofer *et al.*, 2011; Shi and Zhu, 2007). In this study, the fractional factorial design was successfully used for the determination of the significant variables for phytase production. Then, the variables that showed significant effect and contribution to the phytase production were further optimized using FCCCD.

CONCLUSION

The fractional factorial design was successfully applied to identify the significant variables that affecting recombinant phytase production from *E. coli* DH5α. Related to this, seed age (h), inoculum level (%, w/v), L-arabinose concentration (%, w/v), induction time (by measuring cell concentration at 600 nm) was found to be significant ($p<0.05$). Significantly, this indicated that these variables play an important role during cultivations for the recombinant phytase production in *E. coli* DH5α.

ACKNOWLEDGEMENTS

This project was supported by the BIOTEK Malaysia, Ministry of Science, Technology and Innovation, Malaysia

REFERENCES

1. Greiner, R, and Konietzny. U. Phytase for food application. *Food TechnolBiotech.*, 2006; **44**: 125-140.
2. Lei, X.G. and Porres, J.M. Phytase enzymology, applications, and biotechnology. *BiotechnolLett.*, 2003; **25**: 1787-1794.
3. Schumann, W. and Ferreira, L.C.S. Production of recombinant proteins in *Escherichia coli*. *Genet Mol Biol.*, 2004; **27**(3): 442-453.
4. Ries, E.F. and Macedo, G.A. Improvement of phytase activity by a new *Saccharomyces cerevisiae* strain using statistical optimization. *Enzyme Res.*, 2011; 1-6.
5. Zouari, N.F., Haddar, A., Hmidet, N., Frikha, F. and Nasri, M. Application of statistical

experimental design for optimization of keratinases production by *Bacillus pumilus* A1 grown on chicken feather and some biochemical properties. *Process Biochem.*,2010; **45**: 617-626.

6. Vats, P. and Banerjee, U.C. Production studies and catalytic properties of phytases (myo-inositolhexakisphosphatephosphohydrolases): an overview. *Enzyme MicrobTech.*, 2004; **35**(1): 3-14.
7. Li, X.Y., Liu, Z.Q. and Chi, Z.M. Production of phytase by a marine yeast *Kodamaeaohmeri* BG3 in an oats medium: Optimization by response surface methodology. *Bioresource Technol.*, 2008; **99**: 6386-6390.
8. Kaur, P. and Satyanarana, T. Production of cell-bound phytase by *Pichiaanomala* in an economical cane molasses medium: Optimization using statistical tool. *Process Biochem.*, 2005; **40**: 3095-3102.
9. Chakraborty, B., Collins, L.M., Strecher, V.J. and Murphy, S.A. Developing multicomponent interventions using fractional factorial designs. *Stat Med.*, 2009;**28**: 2687-2708.
10. Singh, B. and Satyanarayana, T. Phytase production by a thermophilicmould*Sporotrichum thermophile* in solid state fermentation and its potential applications. *Bioresource Technol.*, 2008a; **99**: 2824-2830.
11. Singh, B. and Satyanarayana, T. Improved phytase production by a thermophilicmould*Sporotrichum thermophile* in submerged fermentation due to statistical optimization. *Bioresource Technol.*, 2008b; **99**: 824-830.
12. Singh, B. and Satyanarayana, T. A marked enhancement in phytase production by a thermophilicmould*Sporotrichum thermophile* using statistical designs in a cost-effective cane molasses medium. *J ApplMicrobiol.*, 2006; **101**: 344-352.
13. Bogar, B., Szakacs, G., Linden, J.C., Pandey, A. and Tengerdy, R.P. Optimization of phytase production by solid substrate fermentation. *J IndMicrobiolBiot.*, 2003a; **30**(3): 183-189.
14. Bogar, B., Szakacs, G., Pandey, A., Abdulhameed, S., Linden, J.C. and Tengerdy, R.P. Production of phytase by *Mucorracemosus* in solid-state fermentation. *BiotechnolProgr.*, 2003b; **19**(2): 312-319.
15. Nuge, T. Production of recombinant Mycobacterium smegmatisphytase in bioreactor. Unpublished M. Sc dissertation, International Islamic University, Malaysia., 2011.
16. Ho, C.W., Chew, T.K., Ling, T.C., Kamaruddin, S., Tan, W.S. and Tey, B.T. Efficient mechanical cell disruption of *Escherichia coli* by an ultrasonicator and recovery of intracellular hepatitis B core antigen. *Process Biochem.*, 2006; **41**: 1829-1834.
17. MeorHussin A.S, Farouk, A-El, Ali, A.M. and Greiner, R. Production of phytate-degrading enzyme from Malaysian soil bacteria using rice bran containing media. *J Agrobiotechnol.*, 2010; **1**(1): 17-28.
18. Heinonen, J.K. and Lahti, R.J. A new and convenient colorimetric determination of inorganic orthophosphate and its application to the assay of inorganic pyrophosphatase. *Anal Biochem.*, 1981; **113**: 313-317.
19. Lee, J., Choi, Y., Lee, P. C., Kang, S., Bok, J., Cho, J. Recombinant production of *Penicilliumoxalicum* PJ3 phytase in *PichiaPastoris*. *World J Microbial Biotechnol.*, 2007; **23**: 443-446.
20. Quan, C.S., Fan, S.D., Zhang, L.H., Wang, Y.J. and Ohta, Y. Purification and properties of a phytase from *Candida krusei* WZ-001. *J BiosciBioeng.*, 2002; **94**(5): 419-425.
21. Pan, H.F., Xie, Z.P., Bao, W.N. and Zhang, J.G. Optimization of culture conditions to enhance cis-epoxysuccinate hydrolase production in *Escherichia coli* by response surface methodology. *BiochemEng J.*, 2008; **42**: 133-138.
22. Sunitha, K., Kim, Y.O., Lee, J.K. and Oh, T.K. Statistical optimization of seed and induction conditions to enhance phytase production by recombinant *Escherichia coli*. *Biochem Eng.*, 2000; **5**(1): 51-56.
23. Carla, J.S.M.S. and Roberto, I.C. Statistical screening method for selection of important variables on xylitol biosynthesis from rice straw hydrolysate by *Candida guilliermondii* FTI 20037. *Biotechnol Tech.*, 1999; **13**: 743-747.
24. Shi, Y., Xu, X. and Zhu, Y. Optimization of Verticilliumlecanii spore production in solid-state fermentation on sugarcane bagasse. *ApplMicrobiolBiot.*,2009; **82**: 921-927.
25. Farliahati, M.R., Ramanan, R.N., Mohamad, R., Puspaningsih, N.N.T. and Ariff, A. Enhanced production of xylanase by recombinant *Escherichia coli* DH5á through optimization of medium composition using response surface methodology. *Ann Microbiol.*, 2010; **60**: 279-285.
26. Pelinski, R., Cerrutti, P., Ponsone, M.L., Chulze, S. and Galvagno, M. Statistical optimization of simple culture conditions to

produce biomass of an ochratoxigenic mould biocontrol yeast strain. *Lett Appl Microbiol*., 2012; **54**(5): 377-382.

27. Nelofer, R., Ramanan, R.N., Raja Abd Rahman, R.N.Z. Basri, M. and Ariff, A.B. Sequential optimization of production of a thermostable and organic solvent tolerant lipase by recombinant *Escherichia coli*. *Ann Microbiol*., 2011; **61**: 535-544.
28. Shi, F. and Zhu, Y. Application of statistically-based experimental designs in medium optimization for spore production of *Bacillus subtilis* from distillery effluent. *BioControl*., 2007; **52**: 845-853.