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Medium Optimization for Phytase Production by Recombinant *Escherichia coli* Using Statistical Experimental Design

CHOI, WON-CHAN, BYUNG-CHUL OH, HYUNG-KWOUN KIM, EUN-SOOK LEE[1], AND TAE-KWANG OH*

Microbial Genomics Laboratory, Korea Research Institute of Bioscience and Biotechnology, P.O. Box115, Yusong, Taejon 305-600, Korea
[1]Department of Preparatory Oriental Medicine, Kyungsan University, Kyungsan 712-240, Korea

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Abstract The production of *E. coli* WC7 phytase from a recombinant *E. coli* strain was optimized using a statistical experimental design approach. Two-level complete factorial designs with seven variables were used for the media optimization. In the first optimization step, the influence of disodium succinate, yeast extract, K_2HPO_4, $NH_4H_2PO_4$, $MgSO_4$, NaCl, and trace elements on phytase production was evaluated. As a result, disodium succinate, yeast extract, $NH_4H_2PO_4$, NaCl, and the trace elements were found to have a positive influence on the phytase production, while K_2HPO_4 and $MgSO_4$ had a negative influence. In the second step, the concentrations of disodium succinate and yeast extract were further optimized using central composite designs. The maximum phytase activity obtained was 234 U/ml using 15.9 g/l disodium succinate, 20 g/l yeast extract, 5 g/l K_2HPO_4, 10 g/l $NH_4H_2PO_4$, 1.5 g/l $MgSO_4$, 4 g/l NaCl, and 1.5 ml/l trace elements, which was about a 14-fold increase in comparison with that obtained using the basal medium.

Key words: Phytase production, *E. coli*, medium optimization, recombinant protein, experimental designs

Phytases (*myo*-inositol hexakisphosphate phosphohydrolases; EC 3.1.3.8 and 3.1.3.26) are enzymes that release phosphate groups from phytic acid [20]. Phytic acid is a *myo*-inositol hexakisphosphate and the main storage form of phosphate in most plant seeds. As such, it is common in animal feedstuff. However, phytic acid cannot be utilized as a phosphate source by nonruminant animals, such as pigs, chickens, and turkeys [8], due to the absence of a phytase enzyme in their digestive tracks. Recently, it was found that the addition of a phytase to animal feed facilitates the rapid degradation and utilization of phytic acid as a phosphate source for monogastric animal growth [20]. In this case, no more inorganic phosphate was required for the animal feedstuffs. As a result, the addition of phytase prevents undesirable phosphate release into the environment [20], thus suggesting that phytases are very important for the prevention of environmental pollution as well as in the prevention of antinutritional effects.

Phytases have already been isolated from various plants and microorganisms. For example, phytases have been found in seeds, such as wheat, barley, beans, corn, and soybeans, while microbial phytases have been found in fungi, in particular *Aspergilli* (*A. ficuum* [7], *A. niger* [18], *A. terreus* [21]), and in bacteria, such as *Aerobacter aerogenes* [9], *Pseudomonas* sp. [4], *Escherichia coli* [10], *Bacillus* sp. [16, 19], and *Enterobacter* sp. [23].

Recently, *E. coli* WC7 phytase was purified and its biochemical properties reported [2]. It was highly stable at an acidic pH and had a relatively high specific activity. Therefore, these properties suggested that it would have potential as an animal-feed additive enzyme.

The productivity of a protein is greatly affected by the nutrient composition of the medium. The optimization of culture media has traditionally been performed by varying one factor while keeping the other factors constant; the one-factor-at-a-time technique. However, this technique is tedious when a large number of factors have to be optimized. Recently, an efficient method was developed to statistically optimize medium components [6, 11, 13-15, 17, 22, 24]. The experimental design is an efficient tool and can be easily adapted to treating problems with a large number of variables.

Accordingly, the goal of the current study was to investigate the effect of the medium on *E. coli* WC7 phytase production and to develop an efficient phytase-production system by statistically optimizing the medium composition.

*Corresponding author
Phone: 82-42-860-4370; Fax: 82-42-860-4595;
E-mail: otk@mail.kribb.re.kr

MATERIALS AND METHODS

Microorganism and Culture Conditions

A genetically modified *E. coli* strain, DH5α (*supE44 ΔlacU169* (φ80 *lacZΔM15*) *hsdR17 recA1 endA1 gyrA96, thi-relA1*), was used as the recombinant host throughout the current work. The plasmid pACYC184 (New England Biolabs, Inc., Beverly, MA, U.S.A.) was used as the cloning vector. The recombinant plasmid, pACEP, harboring the *E. coli* WC7 phytase gene was constructed as shown in Fig. 1.

The *E. coli* cell was transformed with pACEP and grown overnight at 37°C on a detection plate consisting of the following components (l^{-1}); 15 g of D-glucose, 5 g of calcium phytate, 5 g of NH_4NO_3, 0.5 g of $MgSO_4 \cdot 7H_2O$, 0.5 g of KCl, 0.01 g of $FeSO_4 \cdot 7H_2O$, 0.1 g of $MnSO_4 \cdot 4H_2O$, 15 g of agar, and 35 μg/ml of chloramphenicol. A stain-forming clear halo around a colony was selected and used for further study.

The phytase production medium described by Kim and Kim [12] was modified and used as the basal medium for the production of phytase. That is, the basal medium contained the following components (per liter); 10 g of tryptone, 5 g of yeast extract, 5 g of K_2HPO_4, 5 g of NaCl, 0.45 g of $MgSO_4 \cdot 7H_2O$, and 2 ml of a trace element solution containing (per liter); 13.2 g of $CaCl_2 \cdot 2H_2O$, 8.4 g of $FeSO_4 \cdot 7H_2O$, 2.4 g of $MnSO_4 \cdot 4H_2O$, 2.4 g of $ZnSO_4 \cdot 7H_2O$, 0.48 g of $CuSO_4 \cdot 5H_2O$, 0.48 g of $CoCl_2 \cdot 6H_2O$, 0.24 g of $Na_2MoO_4 \cdot 2H_2O$, and 0.06 g of $K_2B_4O_7 \cdot 4H_2O$. The medium was supplemented with 35 μg/ml of chloramphenicol. Based on this basal medium, further stepwise modifications were performed to optimize the phytase production.

The recombinant *E. coli* cultures were grown at 37°C in 500-ml Erlenmeyer flasks containing 100 m of the basal medium on a rotary shaker at 250 rpm.

Phytase Assay

The phytase activity was measured based on the amount of inorganic orthophosphate (P_i) released from phytic acid, using the modified method of Engelen and Heeft [5], with molybdovanadate as the coloring reagent. The reaction mixture contained 1 mM sodium phytate, an appropriate amount of the enzyme, and 100 mM sodium acetate buffer (pH 5.0). The reaction mixture was incubated at 37°C for 10 min. Thereafter, the reaction was stopped by the addition of a color-stop mixture solution (1:1:2 ratio of 80 mM ammonium molybdate: 40 mM ammonium vanadate: 23% nitric acid). One unit of phytase activity was defined as the amount of enzyme required to liberate 1 μmol of P_i per min under the standard assay conditions.

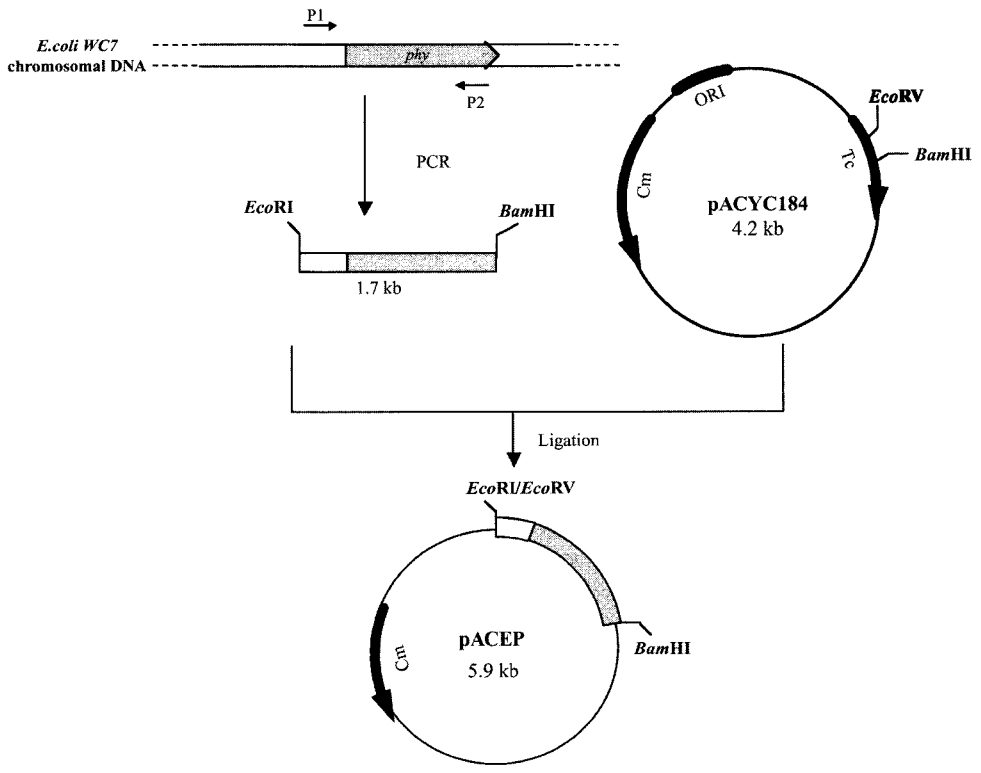

Fig. 1. Construction of the expression plasmid.

pACEP has a 1.7 kb *Eco*RI/*Bam*HI fragment, and it is harboring a precursor form of the *E. coli* WC7 phytase gene and 400 bp of the upstream region. The PCR was performed using two primers. Primer P1 is 5'agg*gaattc*acctgttccccttttgtg3' and P2 is 5'agt*ggatcc*ttacaaactgcacgccggtatgcg3'. Cm, chloramphenicol-resistance gene; Tc, tetracycline-resistance gene.

Experimental Design

Two experimental designs were performed: the first was to determine which components had a major effect on phytase production, while the second was to improve the medium composition and test the influence of other parameters.

A factorial design, one type of experimental design, is very useful in identifying the important nutrients and interactions between two or more nutrients in relatively few experiments as compared to the one-factor-at-a-time technique [3]. Factorial designs require 2^N experiments if N factors have to be investigated. Therefore, in the current case with seven variables, this would mean 128 experiments, which is still a large number. However, the number of experiments can be reduced by using only part of the factorial design (fractional factorial design) without losing any information on the main effects, although some information on the interaction effects is lost [1]. In the current case, the experiments were designed by using the Design Expert program (http://www.statease.com, State Ease Inc., Minneapolis, MN, U.S.A.) and a fractional factorial design was chosen, as the higher-order interactions were expected to be negligible. As such, 1/8 of the 128 experiments were performed, i.e., 16 experiments, and for practical reasons the experiments were arranged in two blocks [3].

Results and Discussion

Preliminary Experiments

A recombinant plasmid, pACEP, was constructed using the pACYC184 vector and *E. coli* WC7 phytase gene containing an upstream 400 bp DNA fragment (Fig. 1). The pACEP was transformed into *E. coli* DH5α cells, and the transformed cells were then used for the production of WC7 phytase in the current experiments.

First, preliminary experiments were performed to obtain approximate estimates on the effects of different medium components on phytase production. The effect of different carbon sources on phytase production was studied by substituting tryptone in the basal medium with various carbon sources. The maximum enzyme production was obtained, when disodium succinate was used as the carbon source (Table 1). In this experiment, 5 g/l yeast extract was added to the basal medium due to the very low phytase activity in yeast extract-free media.

The influence of organic and inorganic nitrogen sources on the enzyme production was also tested. The phytase production level was relatively higher when organic nitrogen sources were used in the medium, except for phytone and polypeptone (Table 2).

The maximum enzyme production was obtained, when yeast extract was used as the nitrogen source. In this case, a total of 10 g/l yeast extract was added to the medium. In addition to basal phosphate sources, ammonium dihydrogen orthophosphate and dipotassium phosphate exhibited a strong positive effect on increasing the enzyme production (data not shown). Magnesium sulfate and sodium chloride were also found to be effective for the enzyme production, along with the minerals present in the trace elements (data not shown). When determining which substrate constituent

Table 1. Effect of carbon sources on cell growth and phytase production.

Carbon source (10 g/l)	Cell growth ($A_{600\ nm}$)	Phytase activity (U/ml)
Glucose	2.66	1.49
Fructose	5.77	3.07
Glycerol	5.33	4.79
Sucrose	3.18	1.29
Lactose	2.93	1.54
Maltose	5.03	2.24
Xylose	2.62	1.65
Sorbitol	5.63	3.96
Mannitol	5.15	4.76
Disodium succinate	5.81	9.80
Trisodium citrate	3.17	1.93
Soluble starch	3.21	2.55
Corn starch	3.45	3.28
Potato starch	3.25	2.13

The basal medium contained 5 g/l yeast extract, 5 g/l K_2HPO_4, 5 g/l NaCl, 0.45 g/l $MgSO_4 \cdot 7H_2O$, and 2 ml/l trace elements. Peptone at 5 g/l was added as the nitrogen source.

Table 2. Effect of nitrogen sources on cell growth and phytase production.

Nitrogen source (5 g/l)	Cell growth ($A_{600\ nm}$)	Phytase activity (U/ml)
None	3.55	2.65
NH_4NO_3	3.73	1.54
$(NH_4)_2SO_4$	3.76	1.97
NH_4Cl	3.91	1.79
KNO_3	4.11	2.46
Urea	4.25	1.65
Malt extract	3.85	2.65
Yeast extract	5.42	3.91
Soytone	4.25	2.55
Phytone	4.37	1.46
Trypticase peptone	4.48	2.15
Proteose peptone No. 3	5.05	1.94
Casein peptone	4.25	2.09
Polypeptone	4.10	1.65
Casamino acid	4.00	2.22
Casein hydrolysate	4.26	1.91
Peptone	3.57	1.84

The basal medium contained 5 g/l yeast extract, 5 g/l K_2HPO_4, 5 g/l NaCl, 0.45 g/l $MgSO_4 \cdot 7H_2O$, and 2 ml/l trace elements. Glucose at 10 g/l was added as the carbon source.

Table 3. Substrate constituents utilized in the first optimization step.

Constituent	Unit	Level	
		Low (-)	High (+)
Disodium succinate (χ_1)	g/l	10	20
Yeast extract (χ_2)	g/l	10	20
K_2HPO_4 (χ_3)	g/l	5	10
$NH_4H_2PO_4$ (χ_4)	g/l	5	10
$MgSO_4$ (χ_5)	g/l	1	2
NaCl (χ_6)	g/l	1	2
Trace elements (χ_7)	ml/l (v/v)	1	2

Table 5. Substrate constituents utilized in the second optimization step.

Constituent	Unit	Level	
		Low (-)	High (+)
Disodium succinate (χ_1)	g/l	20	30
Yeast extract (χ_2)	g/l	15	45
K_2HPO_4 (χ_3)	g/l	3.5	7.5
$NH_4H_2PO_4$ (χ_4)	g/l	7.5	12.5
$MgSO_4$ (χ_5)	g/l	1	2
NaCl (χ_6)	g/l	3	5
Trace elements (χ_7)	ml/l (v/v)	1	2

had a significant effect on phytase production, the various minerals included in the trace elements were regarded as one component rather than as individual components.

Optimization of Medium

In the preliminary experimental step, seven substrate constituents (disodium succinate, yeast extract, K_2HPO_4, $NH_4H_2PO_4$, $MgSO_4$, NaCl, and the trace elements) were chosen for optimization. As described in Materials and Methods, to optimize the medium, a fractional factorial experimental design of the Design Expert program was employed. Each of these constituents was assigned at two concentration levels, so that one optimization step only required sixteen experimental runs to estimate the influence of the constituent level. The coded level of each component concentration is shown in Table 3. To evaluate the effect of each constituent, two coefficients (C_b for biomass and C_p for phytase produced) were calculated. If B is the cell growth ($OD_{600\ nm}$) and P is the phytase production (U/ml), the coefficients C_b and C_p relating to each of the seven constituents are given by:

$$C_{bj}=1/16\times[\Sigma_{i=1}^{16}A_j\times B_j],\ C_{pj}=1/16\times[\Sigma_{i=1}^{16}A_j\times P_i]$$

Here, A_j means either high (+1) or low (-1) level in experimental run i. If a calculated coefficient has a positive value, this means that the constituent had a positive effect at the high level and vice versa.

First Optimization Step

The setup of the first optimization step and the calculated results for C_b and C_p are shown in Table 4. Disodium succinate, yeast extract, and $NH_4H_2PO_4$ all exhibited a positive effect on the phytase production and cell growth. The trace elements had a slightly negative influence on the

Table 4. Design of experimental runs and coefficients for cell growth (C_b) and phytase production (C_p) for each substrate constituent resulting from the first optimization step.

Run	Block	χ_1	χ_2	χ_3	χ_4	χ_5	χ_6	χ_7	Cell growth ($A_{600\ nm}$)	Activity (U/ml)
1	1	-	-	-	-	-	-	-	8.2	37.40
2	2	+	-	-	-	+	-	+	7.6	42.79
3	2	-	+	-	-	+	+	-	10.7	57.40
4	1	+	+	-	-	-	+	+	12.7	72.28
5	1	-	-	+	-	+	+	+	5.4	23.81
6	2	+	-	+	-	+	-	+	6.5	31.18
7	2	-	+	+	-	-	-	+	10.9	76.27
8	1	+	+	+	-	+	-	-	10.5	73.08
9	2	-	-	-	+	-	+	+	9.7	54.59
10	1	+	-	-	+	+	+	-	9.3	60.21
11	1	-	+	-	+	-	-	+	10.0	77.65
12	2	+	+	-	+	-	-	-	11.1	64.68
13	2	-	-	+	+	+	+	-	7.5	40.27
14	1	+	-	+	+	-	-	-	8.1	37.38
15	1	-	+	+	+	-	+	-	10.3	56.13
16	2	+	+	+	+	+	+	+	12.3	73.73
Average									9.4	54.92
C_b		0.34	1.64	-0.49	0.36	-0.45	0.31	-0.29		
C_p		1.99	11.95	-1.43	1.13	-0.74	1.89	1.77		

cell growth, yet positive effect on the enzyme production. A small change in the amount of yeast extract was found to have a strong positive effect on both the cell growth and the phytase production.

Second Optimization Step

For the following experimental step, the dosages of the constituents were changed based on the results of the first optimization step. The concentrations of disodium succinate, yeast extract, $NH_4H_2PO_4$, and NaCl were further increased, K_2HPO_4 was set at an intermediate level, and magnesium sulfate and trace elements were maintained at the same level as in the previous step (Table 5).

The setup of the second optimization step and calculation of the coefficients were performed in the same way as for the first optimization step. The results of the second optimization step and calculated coefficients C_b and C_p are shown in Table 6. In the second optimization step, the phytase production was approximately 2.5-fold (136.87 U/ml) higher compared with the average phytase production of 54.93 U/ml from the first optimization step. From the coefficients given in Table 6, it was concluded that disodium succinate, yeast extract, and $NH_4H_2PO_4$ still had a positive effect on both the cell growth and the phytase production.

The levels of disodium succinate and yeast extract were further optimized using a central composite design (Cochran and Cox, 1957). However, $NH_4H_2PO_4$ was maintained at an intermediate level (center point) because of its precipitation with certain salts, such as $MgSO_4$, and the trace elements at a higher level. The setup of the central composite design and results of this optimization step are shown in Table 7. The levels of the other constituents were set at the level of center point in the second optimization step, since this center point yielded a high production of phytase. In this optimization step, the phytase production was further increased, giving 151.65 U/ml of average value of phytase production.

The best result was obtained with the Run No. 3. The maximum 234.66 U/ml phytase production was obtained. These data suggested that the concentrations of the substrate

Table 7. Substrate constituents and central composite design for two variables in two blocks.

Run	χ_1	χ_2	Cell growth ($A_{600\,nm}$)	Activity (U/ml)
1	−1	−1	11.2	128.67
2	0	0	14.4	126.26
3	−α	0	16.4	234.66
4	0	0	12.6	113.18
5	1	−1	12.1	166.41
6	0	0	11.3	113.41
7	1	1	20.3	159.98
8	0	−α	10.4	102.12
9	−1	1	20.7	195.31
10	0	0	14.2	138.30
11	α	0	15.1	164.80
12	0	α	15.6	136.13
13	0	0	17.2	192.22
Average			14.7	151.65

Table 6. Design of experimental runs and coefficients for cell growth (C_b) and phytase production (C_p) for each substrate constituent resulting from the second optimization step.

Run	Block	χ_1	χ_2	χ_3	χ_4	χ_5	χ_6	χ_7	Cell growth ($A_{600\,nm}$)	Activity (U/ml)
1	1	−	−	−	−	−	−	−	13.2	137.40
2	2	+	−	−	−	+	−	+	12.6	112.79
3	2	−	+	−	−	+	+	−	16.7	147.40
4	1	+	+	−	−	−	+	+	18.7	148.29
5	1	−	−	+	−	+	+	+	10.4	96.81
6	2	+	−	+	−	+	−	+	11.5	103.18
7	2	−	+	+	−	−	−	+	17.9	156.27
8	1	+	+	+	−	+	−	−	17.5	153.08
9	2	−	−	−	+	−	+	+	14.7	124.59
10	1	+	−	−	+	+	+	−	14.3	140.21
11	1	−	+	−	+	−	−	+	20.0	147.65
12	2	+	+	−	+	−	−	−	17.1	157.68
13	2	−	−	+	+	+	+	−	12.5	135.27
14	1	+	−	+	+	−	−	−	13.1	137.38
15	1	−	+	+	+	−	+	−	15.3	136.13
16	2	+	+	+	+	+	+	+	17.3	155.72
Average									15.2	136.87
C_b		0.09	2.39	−0.74	0.36	−0.83	−0.19	−0.04		
C_p		1.67	10.95	−0.18	2.51	−3.74	1.14	−3.86		

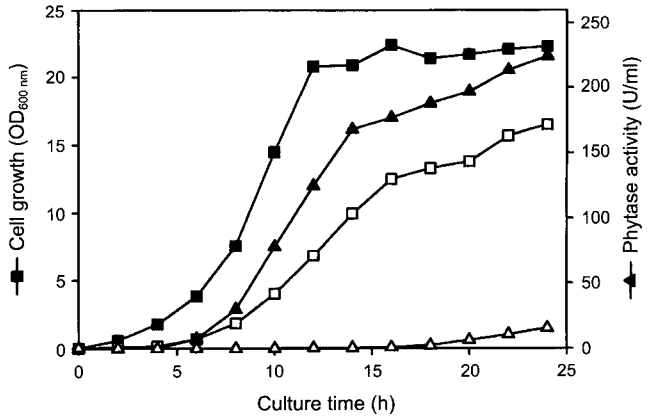

Fig. 2. Time course of phytase production using optimized medium (solid symbols) and basal medium (open symbol) in shake flask: (■) Cell growth; (▲) phytase activity.

constituents in the second optimization step were reasonably optimized. As such, the optimum medium composition derived from the last optimization step was 15.9 g/l disodium succinate, 20 g/l yeast extract, 5 g/l K_2HPO_4, 10 g/l $NH_4H_2PO_4$, 1.5 g/l $MgSO_4$, 4 g/l NaCl, and 1.5 ml/l trace elements. The optimized medium resulted in as much as a 14-fold increase in phytase production when compared to that in the basal medium (Fig. 2).

E. coli WC7 phytase is highly stable at an acidic pH and has higher specific activity than those of *Bacillus* sp. [16, 19], *Aspergillus* sp. [7, 18, 21], and *Enterobacter* sp. [23]. The activity was actually about 10–100 times higher than those of other phytases. Therefore, the phytase from *E. coli* WC7 would appear to have industrial application potential as a feed enzyme. However, since the WC7 phytase is weakly expressed in the wild-type *E. coli* WC7 strain, a pET22b (+) expression vector system was initially used to obtain a large amount of the WC7 phytase. The pET system contained an IPTG-inducible T7 promoter and *E. coli* BL21(DE3) host cell. However, it was found that most of the WC7 phytase protein was produced in an inactive form, as an inclusion body (unpublished data).

Therefore, the current study adapted a pACEP/*E. coli* DH5α system and optimized the culture medium using experimental designs.

Statistically-based experimental designs proved to be valuable tools in optimizing the medium for the phytase production of recombinant *E. coli*. The fractional factorial design used in the first step was an efficient approach to determine which medium components most significantly affected the phytase production. The central composite design used in the last optimization step was then useful in determining the optimum levels for the significant components. When the optimized medium was used, a 14-fold increased productivity (234.66 U/ml) was achieved, which was 317 times higher than that of the wild-type *E. coli* WC7 strain (0.74 U/ml).

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References

1. Box, G. E. O., W. G. Hunter, and J. S. Hunter. 1978. *Statistics for Experimenters.* Wiley, New York, U.S.A.
2. Choi, W. C., B. C. Oh, H. K. Kim, S. C. Kang, and T. K. Oh. 2002. Characterization and cloning of a phytase from *Escherichia coli* WC7. *Kor. J. Microbiol. Biotechnol.* **30:** 1–7.
3. Cochran, W. G. and G. M. Cox. 1957. *Experimental Designs,* 2nd edition. John Wiley and Sons, New York, U.S.A.
4. Cosgrove, D. J. 1970. Inositol phosphate phosphatases of microbiological origin. Inositol phosphate intermediates in the dephosphorylation of the hexaphosphates of myo-inositol, scyllo-inositol, and D-chiro-inositol by a bacterial (*Pseudomonas* sp.) phytase. *Aust. J. Biol. Sci.* **23:** 1207–1220.
5. Engelen, A. J. and F. C. Heeft. 1993. Simple and rapid determination of phytase activity. *J. AOAC Int.* **77:** 761–764.
6. Gawande, B. N. and A. Y. Patkar. 1999. Application of factorial designs for optimization of cyclodextrin glycosyltransferase production from *Klebsiella pneumoniae* AS-22. *Biotechnol. Bioeng.* **64:** 168–173.
7. Gibson, D. 1987. Production of extracellular phytase from *Aspergillus ficuum* on starch media. *Biotechnol. Lett.* **9:** 305–310.
8. Graf, E. 1986. Chemistry and application of phytic acid; An overview. *Phytic acid: Chemistry and Applications,* pp. 1–21.
9. Greaves, M. P., G. Anderson, and D. M. Webley. 1967. The hydrolysis of inositol phosphates by *Aerobacter aerogenes. Biochim. Biophys. Acta* **132:** 412–418.
10. Greiner, R., U. Konietzny, and K. D. Jany. 1993. Purification and characterization of two phytases from *Escherichia coli. Arch. Biochem. Biophys.* **303:** 107–113.
11. Han, J. J. and J. S. Rhee. 1998. Taguchi's robust design method for optimization of lysophosphatidic acid production in an open reactor system. *J. Microbiol. Biotechnol.* **8:** 81–88.
12. Kim, Y. O. and H. K. Kim. 1998. Purification and properties of a thermostable phytase from *Bacillus* sp. DS11. *Enzym. Microb. Technol.* **22:** 2–7.
13. Lee, I. Y., Y. C. Shin, H. N. Chang, Y. H. Park, E. S. Choi, G. J. Kim, and S. W. Nam. 1994. Optimization of fed-batch fermentation for production of poly-β-hydroxybutyrate in *Alcaligenes eutrophus. J. Microbiol. Biotechnol.* **4:** 146–150.

14. Lim, W. J., K. M. Choi, and S. Y. Hwang. 1993. Optimization of an Intact cell system of *Rhodocyclus gelatinosus* KUP - 74 for δ-aminolevulinic acid production. *J. Microbiol. Biotechnol.* **3:** 244–251.
15. Pak, J. H., S. Y. Lee, W. C. Shin, and H. Y. Lee. 1991. Optimization of producing liquid fuel from photosynthetic algal growth. *J. Microbiol. Biotechnol.* **1:** 111–115.
16. Powar, V. K. and V. Jagannathan. 1982. Purification and properties of phytate-specific phosphatase from *Bacillus subtilis*. *J. Bacteriol.* **151:** 1102–1116.
17. Ramirez, J., H. Gutierrez, and A. Gschaedler. 2001. Optimization of astaxanthin production by *Phaffia rhodozyma* through factorial design and response surface methodology. *J. Biotechnol.* **88:** 259–268.
18. Shieh, T. R. and J. H. Ware. 1968. Survey of microorganism for the production of extracellular phytase. *Appl. Microbiol.* **16:** 1348–1351.
19. Shimizu, M. 1992. Purification and characterization of phytase from *Bacillus subtilis* (natto) N-77. *Biosci. Biotechnol. Biochem.* **56:** 1266–1269.
20. Wodzinski, R. J. and A. H. Ullah. 1996. Phytase. *Adv. Appl. Microbiol.* **42:** 263–302.
21. Yamada, K., Y. Minoda, and K. Yamada. 1968. Phytase from *Aspergillus terreus*. Part 1. Production, purification, and some general properties of the enzyme. *Agric. Biol. Chem.* **32:** 1275–1283.
22. Yang, X. M. 1992. Optimization of a cultivation process for recombinant protein production by *Escherichia coli*. *J. Biotechnol.* **23:** 271–289.
23. Yoon, S. J. and Y. J. Choi. 1996. Isolation and identification of phytase-producing bacterium, *Enterobacter* sp. 4, and enzymatic properties of phytase enzyme. *Enzyme Microb. Technol.* **101:** 449–454.
24. Zakaria, M. M., M. Ashiuchi, S. Yamamoto, and T. Yagi. 1998. Optimization for beta-mannanase production of a psychrophilic bacterium, *Flavobacterium* sp. *Biosci. Biotechnol. Biochem.* **62:** 655–660.