Chiang Mai J. Sci. 2019; 46(3) : 431-443
http://epg.science.cmu.ac.th/ejournal/
Contributed Paper

Enhanced Production of Phytase, A Feed Enzyme, from *Pichia kudriavzevii* using Mutagenesis and Improved Culture Conditions

Wanatchaporn Boontham [a], **Natsuda Srivanichpoom** [a], **Pumin Nutaratat** [a,b], **Savitree Limtong** [a,b], **and Nantana Srisuk*** [a,b]

[a] Department of Microbiology, Faculty of Science, Kasetsart University, Chatuchak, Bangkok 10900, Thailand.

[b] Center for Advanced Studies in Tropical Natural Resources, NRU-KU, Kasetsart University, Chatuchak, Bangkok 10900, Thailand.

*Author for correspondence; e-mail: fscints@ku.ac.th

Received: 4 September 2018
Revised Date: 8 February 2019
Accepted: 18 February 2019

Abstract

Pichia kudriavzevii WB17-1, a phytase-producing yeast isolated from duck excrement, was found to produce both cell-bound and extracellular phytases. To enhance extracellular phytase, *P. kudriavzevii* WB17-1 was subjected to induced mutation. Ethylmethane sulfonate (EMS) induced mutation resulted in 2,400 mutants. The mutant *P. kudriavzevii* WB17-1 EMS3 showed the highest extracellular phytase activity. This mutant possessed a 6.2-fold increase in enzyme activity compared to the wild type level. The wild type and mutant were subjected to characterization of *PHYPk*, a gene encoding *P. kudriavzevii* phytase. An open reading frame of 1,071 bp encoding 357 amino acids with a predicted protein molecular mass of 40.056 kDa was identified. To optimize the extracellular phytase activity of *P. kudriavzevii* WB17-1 EMS3, a response surface methodology (RSM) was employed. The highest extracellular phytase activity was obtained in the medium containing 2.95% dextrose and 0.58% peptone with an initial pH of 5.8. The optimized phytase activity was 2.3 times the level obtained under unoptimized conditions and was 14.4 times the wild type level. The results obtained here show successful yeast strain improvement and optimization of extracellular phytase by yeast using RSM.

Keywords phytase, optimisation, mutagenesis, *Pichia kudriavzevii,* yeast

1. Introduction

Phytase (myo-inositol hexakisphosphate phosphohydrolase, EC 3.1.3.8) catalyzes the hydrolysis of phytate, which is a major phosphorus-containing component of oil seeds, cereals and legumes. Phytate is an anti-nutritive factor for monogastric animals including humans and is formed during seed and cereal grain maturation [1]. The anti-nutritional function of phytate is due to its role in the chelation of important cations and some amino groups of the proteins. This negative function of phytate subsequently results in a decrease in the bioavailability of

feed nutrients. Incomplete digestion of phytate is due to a very low level of phytase or the lack of phytase in the digestive tract, resulting in phosphorus deficiency in monogastric animals. The addition of inorganic phosphate to feed supplements will induce an over-accumulation of phosphorus compounds on the farm and thus cause environmental pollution through the eutrophication of water resources [2]. To simultaneously increase the bioavailability of dietary minerals and avoid environmental pollution, supplementation of phytase to animal feed was used. The improvement of phytase production, in particular in microorganisms, is of interest. Microbial phytase production has been found in fungi, bacteria and yeast.

Phytase production by microorganisms has been reported for both submerged and solid-state fermentations. Although enzyme production has been extensively studied and used in solid-state fermentation, it has not yet been flexible enough to be reasonably scaled up for commercial phytase production. Separation and purification of the enzyme still require complicated extraction steps and is a costly process. Furthermore, to avoid heterogeneity in enzyme properties that might occur during solid-state fermentation of phytase, a stirring device is necessary [3]. Submerged fermentation is therefore a successful method to achieve phytase production.

It has been shown that the yeast *Pichia kudriavzevii* genome contains phytase genes, and this yeast has been noted as a phytase producer [4]. Apart from the production of phytase, this yeast has also been reported as a tannase [5], ethanol [4], and D-xylonate-producing yeast [6]. In addition, this yeast exhibited excellent antibacterial activity against several pathogens, i.e., *Enterococcus faecalis, Escherichia coli, Klebsiella* sp., *Pseudomonas aeruginosa, P. alcaligenes* and *Staphylococcus aureus,* by producing killer toxins [7]. Recently, *P. kudriavzevii* was also reported as a potential probiotic [8, 9] showing the possibility of whole cell usage of *P. kudriavzevii*. In this study, we aimed to enhance phytase production in *P. kudriavzevii* through induced mutation with ultraviolet (UV) and ethyl methane sulfonate (EMS) to avoid controversies on using genetic modified organism (GMO). The optimization of phytase production was also carried out using a statistical approach.

2. MATERIALS AND METHODS

2.1 Isolation and Qualitative Screening of Phytase Producing Yeasts

Sixty-one phytate-rich samples such as poultry excrement and poultry intestines were used to isolate phytase-producing yeasts. These samples were diluted and spread onto YPD agar (medium containing 10 g/l yeast extract, 20 g/l peptone, 20 g/l dextrose and 15 g/l agar) supplemented with 2 mg/ml sodium propionate and 1 mg/ml chloramphenicol prior to incubation at 37 °C for three days. Qualitative screening of phytase-producing yeasts was performed by point inoculation on phytase screening (PS) agar (medium containing 3 g/l sodium phytate, 10 g/l glucose, 3 g/l $(NH_4)_2SO_4$, 0.5 g/l $MgSO_4.7H_2O$, 0.5 g/l KCl, 0.1 g/l $CaCl_2$ and trace elements) [10]. The phytase-producing yeasts were identified by the production of a clear zone around the yeast colony on PS agar. The selected yeast isolates were maintained on YPD agar at 4 °C.

2.2 Quantitative Screening of Phytase Producing Yeasts

The phytase-producing yeasts were transferred to 50 ml YPD broth and incubated as seed cultures at 28±2 °C for 18 h on a rotary shaker at 130 rpm. The seed cultures were inoculated into 50 ml of a peptone dextrose (PD) broth (2.75% dextrose and 0.65% peptone, initial pH 5.5) in a 250 ml Erlenmeyer flask and incubated at 37 °C for five days on a rotary shaker at 170 rpm. The culture supernatant was collected by centrifugation at 4427 g for 10 min at 4 °C. Cells

were analyzed for dry weight and cell-bound phytase activity. The supernatant was analyzed for extracellular phytase activity. To determine the yeast cell dry weight, the cell pellet was washed twice with distilled water and dried at 100 °C until a constant weight was obtained.

For cell-bound and extracellular phytase, the activity assay was performed using a modification of the method described by Ullah and Gibson [11]. The reaction mixture, comprising 0.1 ml of 4.5 mM sodium phytate (Sigma, US) solution as the substrate and 0.8 ml of 0.55 M sodium acetate/acetic acid buffer, pH 5.5, was pre-incubated at 37 °C for 5 min. Then, 0.1 ml of crude enzyme or supernatant was added, mixed, and incubated at 37 °C for 20 min and the reaction was stopped immediately by adding 1 ml of 5% trichloroacetic acid (TCA). For the enzyme blank, the reaction mixture contained 0.1 ml of 4.5 mM sodium phytate solution and 0.8 ml of 0.55 M sodium acetate/acetic acid buffer, pH 5.5. Then, 1.0 ml of 5% TCA was pre-incubated at 37 °C for 5 min and 0.1 ml of crude enzyme or supernatant was added, mixed and incubated under the same conditions. The released phosphate was determined spectrophotometrically using Fiske and Subbarow's modified method [12]. The assay tubes containing 2.0 ml of sample and 2.0 ml of freshly prepared acid molybdate reagent (2 volumes of 3% ammonium molybdate, 2 volumes of 11% sulfuric acid solution and 1 volume of 2.7% ferrous sulfate) were measured at A_{700}. The reaction mixture without the enzyme sample was used as a blank. A standard curve of phosphate was made for each independent experiment. One unit of phytase activity was defined as the amount of phytase that liberates 1 μmol of inorganic phosphate per min under assay conditions. All experiments were performed in triplicate and the results presented are the mean values. The highest phytase-producing yeast was selected and used for further study.

2.3 Molecular Identification of Selected Phytase Producing Yeasts

Identification of the yeast strain was based on a comparative analysis of the D1/D2 domain of the large sub-unit (LSU) rRNA gene sequences [13]. The sequence of the D1/D2 region of LSU rDNA was determined from the PCR products amplified from genomic DNA with the forward and reverse primers NL-1 and NL-4, respectively. The amplified DNA was purified with a Gel/PCR DNA fragment extraction kit (Geneaid, Taiwan) in accordance with the manufacturer's manual. The purified PCR product was sequenced (First Base Inc., Malaysia) and analyzed using BLAST.

2.4 Mutagenesis by Ultraviolet (UV) and Ethyl Methane Sulfonate (EMS)

UV and EMS mutagenesis were modified according to Winston [15]. For the analysis of survival rates after UV mutagenesis, the selected phytase-producing yeasts were transferred to a 250 ml Erlenmeyer flask containing 50 ml of YPD broth and incubated at 37 °C for 18 h on a rotary shaker at 170 rpm. The cells were collected by centrifugation at 4500 g for 10 min and washed twice with sterile distilled water. The pellet was suspended in sterile distilled water in order to achieve a 2×10^7 cells/ml cell suspension. One hundred microliters of the cell suspension was plated on YPD agar and exposed to UV radiation (Sylvania G15T8; 254 nm) at a distance of 50 cm for various exposure times (10, 20, 30, 40 and 50 sec). After incubation at 37 °C for 48 h in the dark, the colonies were counted to determine the survival rates. The time that showed 40% survival rates were used for mutation (modified from Winston [14]). The mutants were screened and selected on PS agar and PD broth, respectively.

For analysis of the survival rates after EMS mutagenesis, cells were washed twice and re-suspended in 10 ml of sterile 0.1 M sodium phosphate buffer, pH 7.0, in order to

achieve a 2×10^7 cells per ml suspension. Ten milliliters of the cell suspension was treated with 0.5 ml of EMS (Sigma-Aldrich, Switzerland) and incubated at 37 °C for 0–100 min with a 20 min interval on a rotary shaker at 170 rpm. At each time interval, 1.0 ml of the treated cell suspension was mixed with filter-sterilized 20% sodium thiosulfate to inactivate the EMS. Afterwards, the treated cells were washed twice and re-suspended in an equal volume of sterile distilled water. The cell suspensions were spread on YPD agar and incubated at 37 °C for 24 h. The colonies were counted to determine the survival rates. The mutants were screened and selected on PS agar and PD broth, respectively.

2.5 Phytase Gene Identification and Characterization

To identify the phytase gene of *P. kudriavzevii* (*PHYPk*), the degenerate oligonucleotide primers in contig 396 of the draft genome of *P. kudriavzevii* (GenBank # ALNQ01000395) that contains the phytase gene were used. The *PHYPk* gene was amplified using the PHYF1 primer 5'-GTGCCGACACAGACACAGTATT-3' and PHYR6 primer 5'-GTTGGGTTTGTATCAAGGGTAGT-3' designed using the *PHYPk* gene sequence of *P. kudriavzevii* and analyzed using the primer analysis software NetPrimer (http://www.PremierBiosoft.com/). PCR amplification purification and sequencing were done followed previously method. Alignments were determined using the ClustalW2 program (http://www.ebi.ac.uk/Tools/clustalw2/index.html). The ORF Finder program (http://www.ncbi.nlm.nih.gov/projects/gorf) was used for putative *PHYPk* protein determination. The molecular masses and pIs of the putative proteins were predicted using the compute pI/MW program (http://au.expasy.org/tools/pi_tool.html). The PROSITE database (http://prosite.expasy.org/scanprosite/) was used for Characteristic domains or signature motifs identification.

2.6 Screening of Carbon and Nitrogen Sources for Phytase Production

A yeast inoculum was prepared in YPD broth and incubated on an orbital shaker at 150 rpm at 28 ±2 °C for 18 h. The inoculum was transferred into 50 ml of PD broth and incubated at 37 °C at 170 rpm. Samples were taken daily for seven days. The crude enzyme was collected by centrifugation at 4427 g for 10 min at 4 °C and was analyzed for extracellular phytase activity. The cells were analyzed for their dry weight.

The effects of carbon and nitrogen sources on extracellular phytase production were studied. Four percent by weight of carbon source (glycerol, dextrose, sucrose, galactose, lactose and soluble starch) and 1% (w/v) nitrogen source (yeast extract, malt extract, soy bean extract, peptone, skim milk, corn steep liquor, urea, monosodium glutamate, NH_4Cl and $(NH_4)_2SO_4$) were varied as individual supplements to the production medium. The supernatant was collected and analyzed for extracellular phytase activity. Cells were analyzed for their dry weight.

2.7 Statistical Optimization of Phytase Production

The Box–Behnken design was used to investigate interactions between the factors and to optimize phytase production [15]. In this study, the experimental plan consisted of 46 trials, and the independent variables were coded into three levels: low (− 1), middle (0) and high (+ 1). The experimental design used for the study is shown in supplementary Table 1. The production medium was placed in a 250 ml Erlenmeyer flask containing 50 ml of medium in triplicate and incubated for five days. The data analysis and contour plots were obtained by using Design Expert version 9.0.1. (State-Ease, US). Finally, the model was validated in triplicate.

Table 1 Five factors with coded (-1, 0, +1) and actual values for phytase production by *P. kudriavzevii* WB17-1 EMS3.

Factors	Symbol	Level		
		-1	0	+1
Dextrose (%)	X_1	1.0	2.5	4.0
Peptone (%)	X_2	0.2	0.6	1.0
Temperature (°C)	X_3	35	37	39
Initial pH	X_4	4.0	5.5	7.0
Shaking speed (rpm)	X_5	100	150	200

3. RESULTS AND DISCUSSION

3.1 Isolation and Screening of Phytase Producing Yeasts and Molecular Identification of the Selected Yeasts

One hundred and twenty-two yeasts were isolated from the total of 61 phytate-rich samples, including duck excrement and chicken intestines and excrement. The results of the qualitative screening revealed 93 phytase-producing yeasts with clear zone around their colonies on PS agar. These 93 isolates were then subjected to a quantitative phytase assay as a secondary screening. The results revealed that strain WB17-1 which isolated from duck excrement, had the highest cell-bound and extracellular phytase activities of 26 U/g CDW and 38 mU/ml, respectively, when cultivated in PD broth. Therefore, strain WB17-1 was chosen for identification and strain improvement. The D1/D2 sequence of strain WB17-1 was compared with the sequence of the type strains in the NCBI database. Based on the results of the DNA sequence analysis, strain WB17-1 (accession number MF599089) had 100% similarity with *Pichia kudriavzevii* CBS 5147^T. Thus, strain WB17-1 was identified as *P. kudriavzevii*.

Many studies of yeast phytase indicated that different yeast strains produced different levels of phytase activity. For example, *P. anomala* had a cell-bound phytase activity of 14 U/g CDW when cultivated in PD broth [16], whereas other yeasts (e.g. *Zygosaccharomyces bisporus*, *Schizosaccharomyces octosporus*, *Williopsis saturnus* and *Z. priorionus*) had cell-bound phytase activities of 13, 6, 5, 7 and 10 U/g CDW, respectively, when cultivated in a liquid minimal medium (LMM) [17]. *Saccharomyces cerevisiae*, *P. kudriavzevii*, *P. occidentalis*, *Candida humilis* and *Kazachstania exigua* were isolated from sourdoughs, and they showed extracellular phytase activities in a wide range of 1 to 92 mU/ml [18]. Among phytase producing yeasts, various strains of *P. kudriavzevii* have been reported as good phytase producers. In traditional African foods, *P. kudriavzevii* was identified as a dominant yeast species during the course of phytase-producing yeast isolation and screening [19]. *P. kudriavzevii* TY13, isolated from the Tanzanian fermented food known as togwa, exhibited high phytate degradation and extracellular phytase activity in the range of 35-190 mU/ml in the culture media [20]. *P. kudriavzevii* TY13 was also reported in other work to degrade approximately 95% of the initial phytate in togwa, an African fermented food [21]. The previous research and our research indicate that *P. kudriavzevii* is an attractive yeast species for phytase production. However, the phytase production efficiency of *P. kudriavzevii* appears to be insufficient for large-scale production. Yeast strain improvement using

induced mutation was therefore proposed to avoid the limit of future application of this yeast to feed industry.

3.2 Mutagenesis by Ultraviolet (UV) and EMS (Ethyl Methanesulfonate)

In this study, we enhanced phytase production in *P. kudraivzevii* through induced mutation with UV and EMS. As we did not get the potent mutants those produced higher phytase than that found in the wild type by using UV mutation (total 3,700 UV mutant colonies). We therefore subsequently induced yeast mutation by EMS, one of alkylating agents found to effectively used in yeast strain improvement [22]. In UV-induced mutagenesis, UV exposure time was 10, 20, 30, 40 and 50 sec resulted in 82.22, 55.41, 36.78, 15.04 and 4.31% survival rates, respectively. For EMS mutagenesis, exposure times of 20, 40, 60, 80 and 100 min resulted in 89.06, 64.56, 44.89, 30.10 and 17.87% survival rates, respectively. These results indicated that the exposure times to UV and EMS that yielded a 40% survival rate were 30 sec and 60 min, respectively. Therefore, *P. kudriavzevii* WB17-1 was exposed to UV radiation for 30 sec and to EMS for 60 min. A total of 6,100 mutants were obtained from the UV (3,700 mutant colonies) and EMS (2,400 mutant colonies) treatments. Only three mutants, *P. kudriavzevii* WB17-1 EMS1, *P. kudriavzevii* WB17-1 EMS2 and *P. kudriavzevii* WB17-1 EMS3, had higher levels of phytase activity than the wild type, as determined by the ratio of the diameter and the clear zone of the colony on PS agar. Quantitative assays revealed the maximum cell-bound phytase activity in *P. kudriavzevii* WB17-1 EMS2 (75 U/g CDW), which was up to 2.9 times of the wild type level, whereas *P. kudriavzevii* WB17-1 EMS3 showed the highest extracellular phytase activity (236 mU/ml), up to 6.2 times the wild type level (Table 2).

Table 2 Cell bound (U/g CDW) and extracellular (mU/ml) phytase activities from yeast strains of *P. kudriavzevii* WB17-1 and the mutants in PD broth after 5 days incubation at 37 °C 170 rpm.

Strains	Phytase activities*	
	Cell bound (U/g CDW)	Extracellular (mU/ml)
P. kudriavzevii WB17-1	26 ± 1	38 ± 2
P. kudriavzevii WB17-1 EMS1	40 ± 4	116 ± 5
P. kudriavzevii WB17-1 EMS2	75 ± 3	175 ± 2
P. kudriavzevii WB17-1 EMS3	36 ± 3	236 ± 1

*Results are showed as the mean of three replicated measurements and standard error of the mean.

Mutation by alkylating mutagens creates permanent changes in the character of various organisms. Ethyl methanesulfonate ($CH_3SO_2OC_2H_5$) is a mono-functional alkylating agent that donates its ethyl group. Ethyl methanesulfonate is a very effective and efficient mutagen. The present work suggested that mutation using conventional methods improves the efficiency of phytase production. Qvirist *et al.* showed that the mutant strain of *P. kudriavzevii* TY13 obtained by UV mutation displayed about 8 times higher phytate degradation

than the wild-type strain [23].

For applications, the extracellular enzyme is more favorable to use due to its ease of enzyme harvest and purification compared to intracellular enzyme. In this study, we investigated and focused on optimization of extracellular phytase production of *P. kudriavzevii* WB17-1 EMS3.

3.3 Phytase Gene Identification and Characterization

As determined by nucleotide sequence analysis with contig 396 of the draft genome of *P. kudriavzevii* (GenBank # ALNQ01000395) that contains the phytase gene, the wild type *P. kudriavzevii* WB17-1 exhibited a *PHYPk* gene of 1,071 bp encoding for a putative protein consisting of 357 amino acids (Figure 1), with

```
-296                                TCCAGCAACCCATTGTAGCAAGAACAATGAAATTCAGCGGATAGGTGTTTATCGTCTGG
-237   ATATTTGAGTTTCCACGTGGGAAAACCCGACGTGAAGCTTGGGTATTTCCCTTATTACTAGGTTATTACTTCTCAACAC
-158   GTGGGTTCCTATTACTACAAGGGGGATGTACATCCACTTAACCGTTGTAAAATGGAACATGCCTATAAATAGGGAACAT
-79    CTCTCATCGAAAGTAGCAACAGGTGAAGCAATGAAAACTTTCGAATAACCAAAAGACAGCAACCAAACATAGGATATCA
1      ATG TTG AGT TTA CCA GTT CTG GCC ACC CTA ATC AGT GCA ATT TCT GCA TTA CCA ATT GGT
1       M   L   S   L   P   V   L   A   T   L   I   S   A   I   S   A   L   P   I   G

61     AGT GAT TCA CGT ATT CCG TCT CCA ACA TCT TCA GAG GAT CTC TTC CCC ACT GAT ATT GGT
21      S   D   S   R   I   P   S   P   T   S   S   E   D   L   F   P   T   D   I   G

121    TTC TTT GGT GTA ACA GCC ACC AAT TCT GCA CCT TTT ATG GCA CAA ACA GAC GGT GCA GCT
41      F   F   G   V   T   A   T   N   S   A   P   F   M   A   Q   T   D   G   A   A

181    ATT ACC TAT GGC GTT CCA ACT TCG TCT TAT ACA TTG GCG CAG CCA ATT GAA ACC GCC ATC
61      I   T   Y   G   V   P   T   S   S   Y   T   L   A   Q   P   I   E   T   A   I

241    CAG GCC AAG GAC CAC AGG GAA GGT GAT CAA AAC ATT TTC CAG CTT ATG GGT AAT CTA TCT
81      Q   A   K   D   H   R   E   G   D   Q   N   I   F   Q   L   M   G   N   L   S

301    CCA TAC TAC ATC AGT GAT GGT TGG GGC GTC TAC GAC TAC GCA ATC CCA GGC CAA TGC TCC
101     P   Y   Y   I   S   D   G   W   G   V   Y   D   Y   A   I   P   G   Q   C   S

361    ATC TCA CAA GTC CAT TTG TTA TCA AGA CAT GGT AGT AGA TAT CCA ACA GAT CCG CTG GTT
121     I   S   Q   V   H   L   L   S   R   H   G   S   R   Y   P   T   D   P   L   V

421    ATC GGT AAG TTG CTC GAT AAT AAG AAT TAC ACG GTA ACT GGT GAA TTG GAA TTT TTA AAC
141     I   G   K   L   L   D   N   K   N   Y   T   V   T   G   E   L   E   F   L   N

481    GAT TGG AAA TAT TTA CAA GGT GTT AAT ATT TTA ACC AAG CTA GGT AAC CAA CAA CTA TTT
161     D   W   K   Y   L   Q   G   V   N   I   L   T   K   L   G   N   Q   Q   L   F

541    GAT AAG GGT GTT AGA ACC TTC TTC AGG TAT GGC CAA TTG CAG GAT TGG AAT GAA GAT GCC
181     D   K   G   V   R   T   F   F   R   Y   G   Q   L   Q   D   W   N   E   D   A

601    GAA AAG ATA GTA GCA AGG ACG ACA TCA CAG GAA CGT ATC ACC TCA AGT GCG GAA TAC TTC
201     E   K   I   V   A   R   T   T   S   Q   E   R   I   T   S   S   A   E   Y   F

661    CTT GCA GGG TTC TAT GGT TTG GAC TGG TAT GAC AAG GCC AAT TTG GAA ATT ATT ATT GAA
221     L   A   G   F   Y   G   L   D   W   Y   D   K   A   N   L   E   I   I   I   E

721    TCT AAT GGA TAC AAT GAC ACG CTT GCA CCT TAC GAG CAT TGT CCG CTA ACA GAT AAA ACT
241     S   N   G   Y   N   D   T   L   A   P   Y   E   H   C   P   L   T   D   K   T

781    GAA GCT ACT GCC TCT ATT CCA GCT GAT GGA TTC TTA GAG AAG TAC ACT AAG AAG GCA GTT
261     E   A   T   A   S   I   P   A   D   G   F   L   E   K   Y   T   K   K   A   V

841    GAA AGA CTT AAC AAA CAG ATC AAA GGT TTA ACT TTT AAT GCC AAG AGC ATC CAT CAA ATG
281     E   R   L   N   K   Q   I   K   G   L   T   F   N   A   K   S   I   H   Q   M

901    CAA GAA CTA TGC GCA TAC GAG ACT AAC AAC AAT GGC TTT TCC CAC TTT TGC GGT TTA TTT
301     Q   E   L   C   A   Y   E   T   N   N   N   G   F   S   H   F   C   G   L   F

961    ACT CAA CAA GAA TGG GAG GAC TAT GAA TAT TAT AAC TCT TGG ACT TGG TAC AAC AGC AAT
321     T   Q   Q   E   W   E   D   Y   E   Y   Y   N   S   W   T   W   Y   N   S   N

1021   ATG TTT GGC TCA CCT AAT GGC AGA GCT ATG GGT GCT GGT TGG GTT GAG GAG
341     M   F   G   S   P   N   G   R   A   M   G   A   G   W   V   E   E   *
```

Figure 1 Nucleotide and deduced amino acid sequences of *PHYPk* gene of *P. kudriavzevii* WB17-1. An asterisk (*) indicates stop codon. The deduced TATAA promoter site is underlined (thick line). Amino acid sequence in the gray box show histidine acid phosphatases family signature.

a predicted molecular mass and isoelectric point of pH (pI) of 40.056 kDa and 4.72, respectively. The predicted information for the putative protein appeared to be consistent with the phytases reported from other yeasts and fungi, with molecular masses of 30–100 kDa. At 89 bp upstream of the initiation codon, a putative TATA box (TATAA) was found; however, a CAAT box was not observed (Figure 1). The putative amino acids had a histidine acid phosphatases phosphohistidine signature (ISQVHLLSRHGS), indicating the classification of this phytase to the histidine acid phosphatases family. It should be noted that the amino acid sequence of the wild type strains showed an identical open reading frame to the *PHYPk* gene of the wild type *P. kudriavzevii* WB17-1, *P. kudriavzevii* WB17-1 EMS1, *P. kudriavzevii* WB17-1 EMS2 and *P. kudriavzevii* WB17-1 EMS3 (accession number MF611633, MF611634, MF611635 and MF611636, respectively). These results indicated that the open reading frame of the *PHYPk* gene was not affected by the EMS-induced mutation.

3.4 Screening of Carbon and Nitrogen Sources for Phytase Production

The mutant *P. kudriavzevii* WB17-1 EMS3 showed the highest phytase activity of 232 mU/ml after five days of incubation (Figure 2). The effects of various carbon and nitrogen sources on phytase production are shown in Table 3. The results revealed that, when compared to other mutants, *P. kudriavzevii* WB17-1 EMS3 exhibited the highest extracellular phytase when dextrose and peptone were used as carbon and nitrogen sources, respectively, after five days cultivation at 170 rpm and 37 °C. This result agrees with the report of Vohra and Satyanarayana [16], which indicated that the highest phytase production of yeast *P. anomala* was found in PD broth that compose of dextrose and peptone. However, apart from dextrose, cane juice and cane molasses were also used as potential substrates for phytase production. The yeasts *Zygosaccharomyces bisporus* NCIM 3265, *Z. bisporus* NCIM 3296, *Schizosaccharomyces octosporus* NCIM 3297, *Williopsis saturnus* NCIM 3298 and *Z. priorionus* NCIM 3299 produced more phytase when they were cultured in cane juice and cane molasses media. The activity was 11, 15, 13, 25 and 18 times higher, respectively, than those cultivated in LMM broth that contained dextrose and a few kinds of mineral salts plus 0.2% sodium phytate [17]. These researches indicated that the effect of carbon and nitrogen source to phytase production is dependent on yeast strain. In this study, *P. kudriavzevii* WB17-

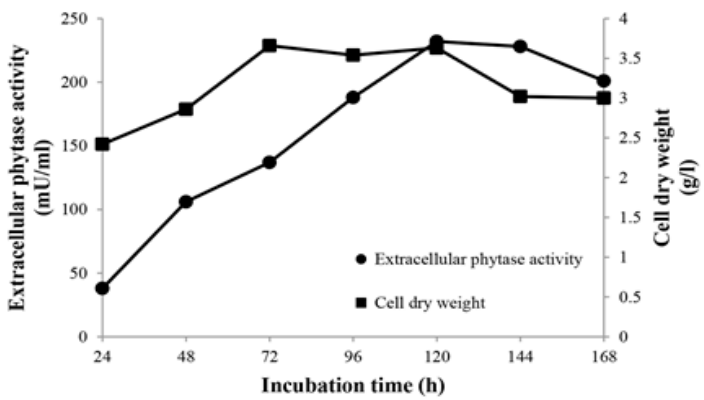

Figure 2 Effect of incubation time on phytase production of *P. kudraivzevii* WB17-1 EMS3 when cultivated in PD broth and incubated at 37 °C and 170 rpm for 168 h.

Table 3 Effect of carbon and nitrogen sources on extracellular phytase production by *P. kudriavzevii* WB17-1 EMS3 when cultivation at 170 rpm and 37 °C for 5 days.

Carbon sources	Cell dry weight (g/l)	Extracellular phytase activity (mU/ml)	Nitrogen sources	Cell dry weight (g/l)	Extracellular phytase activity (mU/ml)
Glycerol	1.97 ± 0.25	-	Yeast extract	5.61 ± 0.15	-
Dextrose	3.62 ± 0.08	228 ± 3.32	Malt extract	0.49 ± 0.02	-
Sucrose	0.53 ± 0.01	-	Soy isolate	4.72 ± 0.26	-
Galactose	0.08 ± 0.02	-	Peptone	3.27 ± 0.15	224 ± 3.78
Lactose	0.13 ± 0.02	-	Skim milk	2.56 ± 0.25	-
Soluble starch	0.11 ± 0.01	-	Corn steep liquor	2.52 ± 0.16	-
			Urea	0.23 ± 0.01	-
			Monosodium glutamate	0.48 ± 0.00	-
			NH_4Cl	0.24 ± 0.01	-
			$(NH_4)_2SO_4$	0.29 ± 0.01	-

1 EMS3 produced more phytase in PD broth than in LMM broth (up to 2.8 times higher rates of production) (data not shown). From this experiment it can be concluded that, dextrose and peptone are the best carbon source and nitrogen source for phytase production by *P. kudriavzevii* WB17-1 EMS3, respectively. Therefore, PD broth composed of dextrose and peptone was chosen as a basal medium for statistical optimization in the next part.

3.5 Statistical Optimization of Phytase Production

The extracellular phytase activity produced by *P. kudriavzevii* WB17-1 EMS3 obtained from the Box–Behnken experimental design is provided in Supplementary Table 1. The phytase activity found in this experiment was varied from 24±1.45 to 553±20.8 mU/ml. To evaluate the RSM model, the observed and predicted values were compared. The two values were close to the median line, indicating that the difference between the actual and predicted values was low (Supplementary Figure 1). Table 4 shows the multiple linear regression of the experimental data. The obtained data fitted well with the model, as suggested by the p value ($p < 0.0001$), the determination coefficient ($R^2 = 0.95$) and the adjusted determination coefficient value (adjusted $R^2 = 0.92$). The closer values between the R^2 and the adjusted R^2 gave a stronger statistical model and the greater correlation between the observed and predicted values [24].

The effects of dextrose, peptone, temperature, initial pH and shaking speed on phytase activity were predicted by the model:

$$Y = -62293.54019 + 628.91793X_1 + 85.62925X_2 + 3230.25276X_3 + 1126.63703X_4 - 8.05362X_5 + 83.75000X_1X_2 - 11.83333X_1X_3 + 34.77778X_1X_4 + 0.27333X_1X_5 + 48.43750X_2X_3 - 41.25000X_2X_4 + 0.32500X_2X_5 - 8.25000X_3X_4 + 0.71289X_3X_5 - 0.093333X_4X_5 - 82.97247X_1^2 - 1655.86292X_2^2 - 44.75478X_3^2 - 76.93544X_4^2 - 0.060808X_5^2$$

Table 4 Analysis of variance (ANOVA) in the regression model for optimization of phytase production by *P. kudriavzevii* WB17-1 EMS3.

Source	Sum of Squares	Degree of freedom	Mean Square	F-value	Prob > F
Model	1.128E+006	20	56380.11	25.43	< 0.0001
A-dextrose	54640.71	1	54640.71	24.64	< 0.0001
B-peptone	294.03	1	294.03	0.13	0.7188
C-temp	26385.53	1	26385.53	11.90	0.0020
D-pH	43882.03	1	43882.03	19.79	0.0002
E-rpm	8225.63	1	8225.63	3.71	0.0655
AB	10100.25	1	10100.25	4.56	0.0428
AC	5041.00	1	5041.00	2.27	0.1441
AD	24492.25	1	24492.25	11.05	0.0027
AE	1681.00	1	1681.00	0.76	0.3922
BC	6006.25	1	6006.25	2.71	0.1123
BD	2450.25	1	2450.25	1.11	0.3032
BE	169.00	1	169.00	0.076	0.7848
CD	2450.25	1	2450.25	1.11	0.3032
CE	16329.49	1	16329.49	7.36	0.0119
DE	196.00	1	196.00	0.088	0.7687
A^2	3.023E+005	1	3.023E+005	136.34	< 0.0001
B^2	6.088E+005	1	6.088E+005	274.58	< 0.0001
C^2	2.649E+005	1	2.649E+005	119.49	< 0.0001
D^2	2.599E+005	1	2.599E+005	117.22	< 0.0001
E^2	1.911E+005	1	1.911E+005	86.16	< 0.0001
Residual	55432.70	25	2217.31		
Lack of Fit	54835.37	19	2886.07	28.99	0.0002
Pure Error	597.33	6	99.56		
Total	1.183E+006	45			
$R^2 = 0.95$, adj $R^2 = 0.92$					

where Y is phytase activity (mU/ml), X_1 is dextrose, X_2 is peptone, X_3 is temperature, X_4 is initial pH, and X_5 is shaking speed.

A contour plot exhibited the interactions among dextrose, peptone, temperature, initial pH and shaking speed on phytase activity (Figure 3). Among the variable interactions, the interaction between dextrose and peptone, dextrose and initial pH, temperature and shaking speed were shown to significantly ($p \leq 0.05$) affect phytase production by *P. kudriavzevii* WB17-1 EMS3. The optimum condition for phytase production was 2.95% dextrose, 0.58% peptone, 37 °C, initial pH 5.8 and shaking speed 160 rpm. This

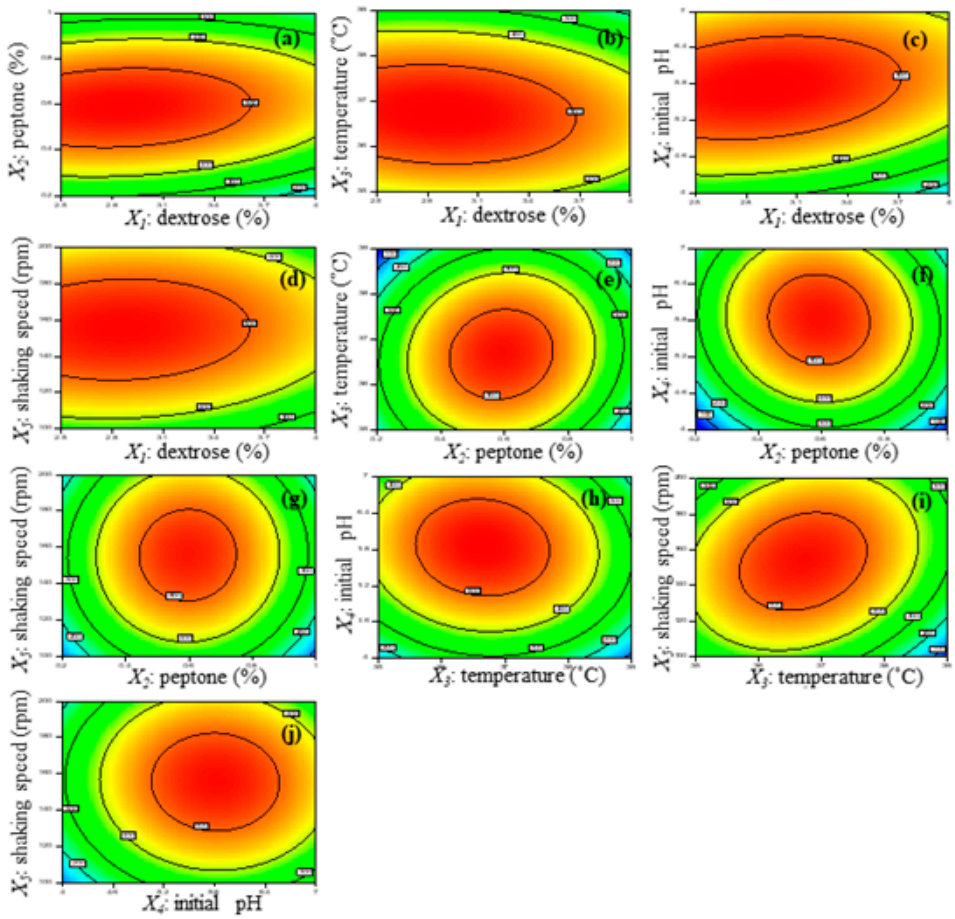

Figure 3 Contour plot of interactions term of dextrose and peptone (a), dextrose and temperature (b), dextrose and initial pH (c), dextrose and shaking speed (d), peptone and temperature (e), peptone and initial pH (f), peptone and shaking speed (g), temperature and initial pH (h), temperature and shaking speed (i) and initial pH and shaking speed (j) on phytase production by *P. kudriavzevii* WB17-1 EMS3 using Box-Behnken experimental design.

condition was used in validation test and results showed that the observed value of phytase activity (545.37±6.70 mU/mL) was close to the predicted activity of phytase (553.37mU/mL). The root mean square error (RMSE) value of our model was 11.31 (2.04%), which proved the correctness of this model. Furthermore, a good correlation between the predicted and observed values was exhibited, indicating that the empirical model can be used to adequately describe the relationship between the factors and phytase production.

In RSM analysis, the contour plot showed a medium level for all factors that enhanced phytase production. The RSM analysis showed that phytase activity was increased 2.3-fold compared to production under unoptimized media and conditions. Ries and Macedo [25] also used statistical optimization to enhance phytase production by *Saccharomyces cerevisiae* and the results showed a 10-fold increase of phytase activity. Li *et al.* enhanced phytase

production in *Kodamaea ohmeri* BG3 by using statistical optimization and obtained 9-fold enhancement [26]." Puppala *et al.* [27] investigated phytase producing ability of *Candida tropicalis* NCIM 3321 and enhanced phytase production by four folds (from 0.46 to 1.95 U/ml). This study achieved a total 14.4-fold improvement in phytase production. This indicates that the process of improvement and optimization in the present research has potential and was successfully performed when compared to the other research described above.

4. Conclusions

EMS mutagenesis improved phytase production in *P. kudriavzevii* WB17-1. The mutant *P. kudriavzevii* WB17-1 EMS3 was obtained by EMS-induced mutation that did not affect the open reading frame of the *PHYPk* gene. The mutant showed a tendency to be a good phytase producer in basic medium that was at its most optimal when it consisted of 2.95% dextrose and 0.58% peptone and had an adjusted initial pH of 5.8. The overall 14.4-fold improvement in phytase production was achieved by mutation and the optimization process. The results obtained here show successful yeast strain improvement, using non-recombinant DNA technology, and optimization of extracellular phytase production by yeast. The enzyme will be used in feed premix production.

Acknowledgments

This work was supported by the Kasetsart University 72th Year Anniversary Graduate Scholarship through the Graduate School, Kasetsart University to W. Boontham (2014-2016) and Kasetsart University Research and Development Institute (KURDI) to N. Srisuk (2012-2013). The authors would also like to thank the Higher Education Research Promotion and National Research University Project of Thailand, Office of the Higher Education Commission.

Conflicts of interests

The authors declare that they have no conflict of interest in the publication.

References

[1] Konietzny U. and Greiner R., *Int. J. Food Sci. Technol.*, 2002; **37:** 791-812. DOI 10.1046/j.1365-2621.2002.00617.x.

[2] Raboy V. *Trends Plant Sci.*, 2001; **6:** 458-462. DOI 1016/S1360-1385(01)02104-5.

[3] Coban H.B. and Demirci A., *Bioproc. Biosyst. Eng.*, 2014; **37(4):** 609-616. DOI 10.1007/s00449-013-1028-x.

[4] Chan G.F., Gan H.M., Ling H.L. and Rashid N.A.A., *Eukaryot. Cell*, 2012; **11:** 1300-1301. DOI 10.1128/EC.00229-12.

[5] Mandal S. and Ghosh K., *Food Biotechnol.*, 2013; **27(1):** 86-103. DOI 10.1080/08905436.2012.755929.

[6] Toivari M., Vehkomäki M.L., Nygård Y., Penttilä M., Ruohonen L. and Wiebe M.G., *Bioresour. Technol.*, 2013; **133:** 555-562. DOI 10.1016/j.biortech.2013.01.157.

[7] Bajaj B.K., Raina S. and Singh S., *J. Basic Microbiol.,* 2013; **53(8):** 645-656. DOI 10.1002/jobm.201200187.

[8] Greppi A., Saubade F., Botta C., Humblot C., Guyot J.P. and Cocolin L., *Food Microbiol.*, 2017; 62: 169-177. DOI 10.1016/j.fm.2016.09.016.

[9] Saber, A., Alipour B., Faghfoori Z., Mousavi jam A. and Yari Khosroushahi A., *Nutr. Res.*, 2017; 41:36-46. DOI 10.1016/j.nutres.2017.04.001.

[10] Lambrechts C., Boze H., Moulin G. and Galzy P., *Biotechnol. Lett.,* 1992; **14(1):** 61-66. DOI 10.1007/BF01030915.

[11] Ullah A.H.J. and Gibson D.M., *Prep. Biochem.*, 1987; **17(1):** 63-91. DOI 10.1080/00327488708062477.

[12] Fiske C.H. and Subbarow Y., *J. Biol. Chem.*, 1925; **66:** 375-400. DOI 10.1021/i560130a017.

[13] Kurtzman C.P. and Robnett C.J., *Antonie van Leeuwenhoek,* 1998; **73(4):** 331-371.

[14] Winston F., EMS and UV Mutagenesis in Yeast; in Ausubel F.M. ed., *Current Protocols in Molecular Biology,* John Wiley and Sons, New York, 2008: 13.3B.1-13.3B.5.

[15] Box G.E.P. and Behnken D.W., *Technometrics*, 1960; **2(4):** 455-475. DOI 10.1080/00401706.1960.10489912.

[16] Vohra A. and Satyanarayana T., *Biotechnol. Lett.*; 2001; **23(7):** 551-554. DOI 10.1023/A:1010314114053.

[17] Pable A., Gujar P. and Khire J.M., *J. Food Biochem.*, 2014; **38(1):** 18-27. DOI 10.1111/jfbc.12020.

[18] Nuobariene L., Hansen Å.S. and Arneborg N., *LWT-Food Sci. Technol.*, 2012; **48:** 190-196. DOI 10.1016/j.lwt.2012.03.011.

[19] Greppi A., Krych Ł., Costantini A., Rantsiou K., Hounhouigan D.J., Arneborg N., Cocolin L. and Jespersen L., *Int. J. Food Microbiol.*, 2015; **205:** 81-89. DOI 10.1016/j.ijfoodmicro.2015.04.011.

[20] Hellström A., Qvirist L., Svanberg U., Vilg J.V. and Andlid T., *J. Appl. Microbiol.*, 2015; **118:** 1126-1136. DOI 10.1111/jam.12767.

[21] Hellström A.M., Almgren A., Carlsson N.G., Svanberg U. and Andlid T.A., *Int. J. Food Microbiol.*, 2012; **153:** 73-77. DOI 10.1016/j.ijfoodmicro.2011.10.018.

[22] Mobini-Dehkordi, M., Nahvi I., Zarkesh-Esfahani H., Ghaedi K., Tavassoli M. and Akada R., *J. Biosci. Bioeng.*, 2008; **105(4):** 403-408. DOI 10.1263/jbb.105.403.

[23] Qvirist L., Vorontsov E., Vilg J.V. and Andlid T., *Microb. Biotechnol.*, 2017; **10(2):** 341-353. DOI 10.1111/1751-7915.12427.

[24] Myers R.H. and Montgomery D.C., *Response Surface Methodology: Process and Product Optimization using Designed Experiments,* Wiley, New York, 1995.

[25] Ries E.F. and Macedo G.A. *Enzyme Res.*, 2011; DOI 10.4061/2011/796394.

[26] Li, X., Chi Z., Liu Z., Yan K. and Li H. *Appl. Biochem. Biotechnol.*, 2008; **149(2):** 183-193.

[27] Puppala K.R., Naik T., Shaik A., Dastager S., Kumar V.R., Khire J. and Dharne M., *Biocatal. Agric. Biotechnol.*, 2018; **13:** 225-235. DOI 10.1016/j.bcab.2017.12.013.