

## Site Directed Mutagenesis to Improve *E. Coli* Phytase Activity for Animal Feed

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**Abstract:** Phytate is largely unavailable to monogastric animal such as swine, poultry and fish, as they lack of sufficient endogenous enzymatic activity to hydrolyze phytate. The result is the elimination of precious nutrients that would be beneficial to their growth; furthermore, they will excrete most of the indigestive phytate which can contribute to phosphorus being over applied to the land. Phosphorus has a beneficial impact on vegetative growth on land as well as marine vegetation, causing an increased growth of weeds. This enhanced vegetation consumes large amounts of oxygen, resulting in the loss of aquatic life and ultimately contributes to water pollution and eutrophication of ground water and aquatic environment. Phytase, a type of histidine acid phosphatase hydrolyzes phytin phosphorus and when present in an animal's digestive tract, benefits the animal while reducing total phosphorus levels in manure. Computer modeling has been used to identify and examine the active site of phytase. The factors influencing the ligand binding strength in the active site were analyzed and computational site directed mutagenesis experiments were carried out to evaluate the effects of mutations on the binding strength before and after mutation. From the directive results of computational studies, point mutation was introduced by site directed mutagenesis using polymerase chain reaction (PCR). The activity was measured by kinetic characterization with phytate as a substrate. Decrease in  $K_M$  notable in all functional mutants indicates that all mutant shows increment in substrate binding. Two functional mutants showed improvement in phytase activity and thermostability.

**Key words:** *Phytase; Phytic acid; E. coli phytase; Site directed mutagenesis.*

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### INTRODUCTION

Phytase is a type of histidine acid phosphatase (EC 3.1.3.8) (myo-inositol hexakisphosphate phosphohydrolase) that catalyzes phosphate monoester hydrolysis of phytic acid in a stepwise manner into myo-inositol and inorganic phosphate (Kim, M.S. and X.G. Lei, 2008). Phytic acid generally occurs as a complex of calcium, magnesium and potassium salt and proteins. It is the predominant form of phosphorus in cereals, oil seed and legumes and account for 3% of the seed dry weight and 60 to 90% of the total phosphorus present in seeds (Graf, E., 1986). Animals such as swine, poultry and fish that lack of significant enzymatic activity to hydrolyze phytate cause most of the phytate to be excreted to the environment thus polluting the land (Boyce, A. and G. Walsh, 2007). Accumulation of phytate phosphorus in manure poses a serious pollution problem, contributing to eutrophication of surface water area of the world where the monogastric live stock production is intensive.

Bacterial phytase such as *Pseudomonas* sp., *Bacillus* sp., *Raoultella* sp., *Escherichia coli*, *Citrobacter braaki*, *Enterobacter* and anaerobic ruminant bacteria are the best alternative to fungal sources to produce the enzyme (Konietzny, U. and R. Greiner, 2004). *Escherichia coli* phytase has the promising value for industrial application due the high phytase specific activity and its resistance to proteolytic degradation (Greiner, R., 1993). Recent advances in biotechnology may revolutionize the commercial enzyme industry by offering alternative, cost effective methods of enzyme production. Application of recombinant DNA technology has enable manufacturer to increase the yield and efficiency of enzyme production and to create new products. The original source organism need no longer limit the production of commercial enzymes.

Computational design benefits from the ability to search a large space than possible by purely experimental methods and is well suited to designing novel functions (Bolon, D.N., 2002). In previous work we used computational design approaches to examine and improve the binding strength of an *E. coli* phytase isolated from local waste water bacterium. Based from the computational design results, we are now generating mutant enzymes and to characterize these mutant enzymes and to compare the results for both experiments: *in silico* and 'wet-lab'.

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## MATERIALS AND METHODS

### **Chemicals:**

A local *Escherichia coli* strain from Malaysian waste water was obtained as described in the literature (Greiner, R., 2007). Polymerase chain reaction (PCR) was performed on an Eppendorf Gradient Mastercycler. *Pfu* DNA polymerase was purchased from Finnzyme Diagnostic and Ni-NTA agarose as well as other reagents were purchased from Qiagen. All restriction enzymes were purchased from New England Biolabs. Primers used for PCR were synthesized by Next Gene Scientific. Both cloning vector and expression vector were purchased from Invitrogen.

### **Primer sequence and PCR conditions:**

The nucleotide sequence of the flanking primers for forward and reverse were (F1) 5'-CATACCCATGGGCTCTGGATCC-3' and (R1) 5'-GTTATCGCTTAGCCGACGCC-3' respectively. The flanking primers were designed to introduce unique restriction sites namely *Nco I* and *Blp I* for identification of the mutation by restriction enzyme digestion. The mutagenic primers (M1) 5'-GCATCACCGTCTGACGGAG-3'; (E1) 5'-CTGACGCGGATATTTCTCCTGC-3' and (F1) 5'-CGCGCAAGAGTATTTGCTACAAC-3' each pair of the mutagenic primer were designed to introduce one mutation of the *E. coli* phytase gene. The first PCR mixture contained 1-10 nM of DNA fragments as a template, 10 pmol of each of a mutagenic primer and each of the flanking primers, 10 nmol of dNTP's and *pfu* DNA polymerase in a total volume of 50  $\mu$ l. The thermal cycling conditions were: 94 °C for 15 min, 30 cycles of amplification (94 °C, 1 min; 45 °C, 1 min; 72 °C, 3 min) followed by 72 °C for 10 min and holding at 4 °C. In the second PCR reaction, 2  $\mu$ l of the purified products from the first PCR reaction were used as templates and megaprimer (Xie, Z.H., X.J. Shi, 2009). Primers used as for the second reaction were the flanking primers that were designed and equipped with unique restriction sites. Same amounts of PCR reagents as well as the thermal cycling conditions were performed.

### **Kinetic study:**

The initial velocity region of the enzymatic reaction needs to be determined and subsequent experiments were conducted in the linear range, where less than 10% of the substrate has been converted to product. To retain linearity during the course of experiments enzyme concentrations were modified appropriately. Product was measured at certain time using standard phytase assay at various concentration of Ni-NTA agarose purified enzyme (results not shown) and one substrate concentration. Once the initial velocity condition was established, the substrate concentrations were varied from 0.05 mM to 0.5 mM to generate saturation curve to determined  $K_M$  values. The enzymatic reactions were initiated by mixing the 100 mM sodium phosphate and different concentration of substrate from the pre-determined range (0.05-0.5  $\mu$ M). Reaction was timed once enzyme (0.04  $\mu$ g) was added to the reaction mixture that was incubated at 37 °C. Aliquot (40  $\mu$ l) was taken from the reaction mixture every 20 seconds for 10 minutes and the aliquot was added to a prepared stop solution in 96-well micro plate. The product generated was plotted against the reaction time. Initial velocity ( $v_0$ ) for each substrate concentration reaction was calculated from the slope of the reaction progress curve. The resulting slopes were plotted on the Y-axis versus the concentration of the (X-axis). Kinetic constants were calculated by nonlinear least-square regression, fitting the data to the Henri-Michaelis –Menten equation.

### **Thermal stability:**

Thermal stability for the Ni-NTA agarose purified mutant enzymes were measured by incubating the enzymes at certain temperature from 30-90 °C for 30 min and were placed on ice after the incubation period for 5 min. Standard phytase enzyme assays were performed to measure the residual activity of the enzyme after being exposed to the certain temperature for 30 minutes. The relative enzyme activity was then plotted against temperature.

Thermodynamic stability ( $T_m$ ) was measured by differential scanning calorimetry (DSC). Ni-NTA agarose purified enzyme samples were lyophilized (Labcono, Missouri, USA) and tested for DSC using a Mettler Toledo (Columbus, USA). Temperature was in the range of 30-90 °C at a scan rate of 10 °C.min<sup>-1</sup> on 5 mg samples.

## RESULT AND DISCUSSION

### **Computer simulation:**

From previous molecular modeling study, mutation at residue number 216 from methionine to arginine and at position 219 from glutamic acid to arginine decreases in docking score by about -20.00 kcal/mol. The score indicates the favorability of the ligand to bind to the active site region. More negative score implies better binding. The scores suggest that the ligand bind stronger to the mutated structure compared to the structure before mutation.

In this study we combine all mutations that are expected to introduce good binding strength by enhancing docking score, hydrogen binding and accessible surface due to mutual hydrogen donor or acceptor surface and hydrophobic interaction. Below is the table that list two set of single mutations and triple, one set of double mutations and the wild type structure so that improvement in the binding score and the additional and reduction of the number of hydrogen bonds can be compared.

**Table 1:** Binding score and number of hydrogen bond between ligand and contact residue.

Structure	Hydrogen bond	Score (kcal/mol)
Wild type	12	-202.611±4.73
M216R	16	-223.601±6.974
E219R	13	-216.468±3.648
E219R/M216R	15	-234.95±9.27
M216R/E219R/F254E	16	-220.681±11.33
K65E/K97M/S209G	12	-218.601±5.92

From the results presented in Table 1, the binding scores for M216R and E219R mutations were expected to improve binding strength as well as multiple mutations of M216R/E219R/F254E and E219R/M216R. These are mutations occurring in the active site region of the enzyme except for K65E/K97M/S209G which is not in the active site region. Mutations at positions K65E/K97M/S209G have been reported to give increment in catalytic activity and approximately 20% improvement in thermostability at 80 °C for 10 minutes (Kim, M.S. and X.G. Lei, 2008). Theoretically, the designed mutations give slightly increase in binding score from -202.611 kcal/mol to -207.117 kcal/mol. Besides docking score as the indicator to the ligand binding strength, the number of additional of hydrogen bonds between the ligand and residues before and after mutation also can be analyzed as one of the factors that contribute to the better ligand binding. Compared with the wild type structure, all five introduced mutations showed enhanced docking score which will result in improvement in binding strength and may also improve the catalytic activity of the mutated enzyme.

#### Identification of the PCR product:

First round PCR generated two DNA fragments (megaprimer), each produced from mutagenic primer pair together with appropriate flanking primer. The electrophoretic analysis of the PCR product gives a single band with the expected fragment size (results not shown). After PCR purification step, the two products were used as megaprimer for the second PCR reaction that generates full length of blunt mutated fragment. The fragments were then cloned into Zero Blunt TOPO cloning vector.

#### Mutant screening and sequencing:

The overnight cultures of mutant phytase gene that grow on LB/amp agar plates were screened for the gene insertion in the vector by colony PCR. The colony PCR was performed using flanking primers and the results of the agarose gel electrophoresis would be the size of the whole mutated gene fragment which is 1004 bp. Plasmids from colonies that showed correct fragment size were extracted and subjected to restriction enzyme digestion analysis for confirmation. The recombinant plasmids were identified by digestion with *Nco I* and *Bln I*. The products of the digestion were then analyzed by agarose gel electrophoresis. Recombinant plasmids that gave positive results from colony PCR and endonuclease digestion were sent for DNA sequencing.

#### Kinetic study:

Steady-state kinetic constants for the different mutation of *E. coli* phytase were determined for phytate in the 50-500  $\mu\text{M}$  range using standard acid phosphatase assay. Prior to the determination of the kinetic constant, it was confirmed that introduction of the amino acid substitutions in the different mutant did affect the substrate binding in the active site at ES stage, thus affecting the catalytic activity of the enzyme. Table 2 provides a summary of the kinetic data.

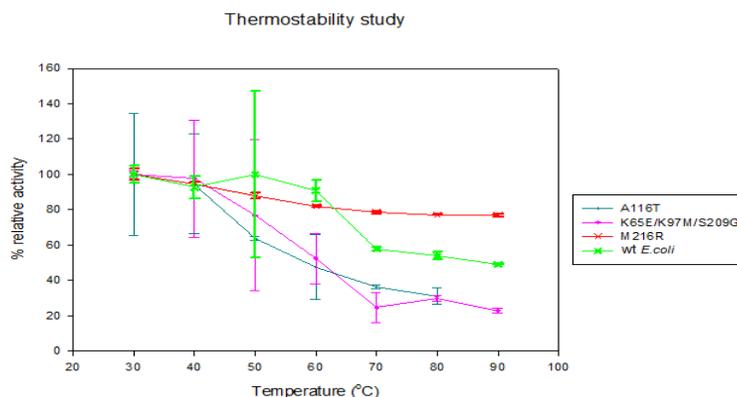
**Table 2:** Enzyme kinetic parameters for the hydrolysis of phytate by Ni-NTA agarose purified phytase.

Mutant	$K_M$ ( $\mu\text{M}$ )	$V_{\max}$ ( $\mu\text{M}/\text{min}$ )	$k_{\text{cat}}$ ( $\text{sec}^{-1}$ )	$\frac{k_{\text{cat}}}{K_M}$ ( $\text{M}^{-1}\text{sec}^{-1}$ )
Recombinant WT <i>E. coli</i>	147.22	0.228	$1.717 \times 10^3$	$1.166 \times 10^7$
M216R	45.102	0.1476	$1.111 \times 10^3$	$2.463 \times 10^7$
A116T	106.926	0.324	$2.440 \times 10^3$	$2.282 \times 10^7$
K65E/K97M/S209G	124.176	0.1382	$1.041 \times 10^3$	$8.383 \times 10^6$
H17A	-	-	-	-
E219R	-	-	-	-
M216R/E219R	-	-	-	-
M216R/E219R/F254E	-	-	-	-

For the presumed natural substrate of *E. coli* phytase which is phytate, out of seven mutants, four mutants turned out to be inactive. One of the mutants, H17A, was expected to be inactive and purposely mutated for validation to the computer modeling. Whereas all other functional mutants studied, showed decreased in Michaelis constant  $K_M$  significantly and the two mutants M216R and K65E/K97M/S209G are in agreement to the computer simulation results. A116T showed decreased in  $K_M$  and notably highest increase in  $k_{cat}$  value. Thus, this mutant showed an increase in catalytic efficiency,  $k_{cat}/K_M$ , as compared to wild type enzyme. In accordance with the result obtained, the M216R and K65E/K97M/S209G mutants showed lower  $K_M$  values, whereas  $k_{cat}$  is hardly affected by the mutations but M216R showed a slight increment in catalytic activity.

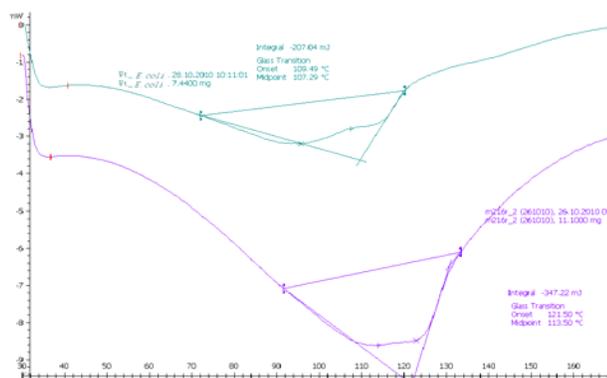
**Thermostability study:**

Wild type *E. coli* phytase enzyme and its mutants were investigated by incubating the enzyme over a period of time at different temperatures. M216R displayed the highest residual activity with 28% improvement over that of the wild type enzyme after being heated at 90 °C for 30 minutes (Figure 1). Because this M216R mutant was promising, its melting temperature was determined by Differential Scanning Calorimetry (DSC). The midpoint of thermal unfolding ( $T_m$ ) was increased by 15.51 °C as compared to the wild type enzyme (Figure 2).



**Fig. 1:** Residual activity of Ni-NTA agarose purified wild type and mutant phytase in thermostability study.

Based on structural prediction to probe molecular basis of thermostability, the substitution of M216R introduces four additional hydrogen bonds between ligand and active site residues. The newly introduced hydrogen bonds might play an important role in stabilizing the interactions between ligand and active site which could contribute to overall stability of the mutant enzyme. The newly formed hydrogen bonds are predicted to stabilize the local structure and then improve the enzyme thermostability.



**Fig. 2:** DSC curve and melting temperature of Ni-NTA agarose purified wild type (green) and M216R mutant (purple).

The increase in hydrogen bond has been suggested as one of the principle determinants of enhanced thermal stability (Kumar, S., 2000). As for other mutants, the residual activity dropped approximately 30 °C as compared to wild type at 90 °C for 30 minutes.

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