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Highly Active $F^{260}R/T^{312}R$ Double Mutant Phytase from *Yersinia Intermedia* for the Efficient Hydrolysis of Phytic Acid

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ABSTRACT

Phytase hydrolyzes phytic acid. Phytic acid is the main form of phosphorus storage in plant seeds in cereals, legumes, and oilseeds. In addition, phytic acid chelates essential minerals and binds to amino acids and proteins and prevents the action of digestive enzymes leads to decrease in the digestibility of proteins. Monogastric animals are unable to hydrolyze phytic acid due to the lack of phytase in their digestive system. In the current study, based on the docking results and DynaMut webserver predictions, $T^{312}R$ and $F^{260}R$ mutants, were chosen. Then, using Quick-Change PCR, the two residues in the active site of phytase from *Yersinia intermedia* phytase were mutated to Arg. Moreover, a double mutant $F^{260}R/T^{312}R$ was generated. Afterwards, the recombinant mutant and wild-type phytases were expressed in bacterial host, purified and their activities were measured. According to the results, the activities of $T^{312}R$ and $F^{260}R$ almost remained unchanged; however, that of $T^{312}R/F^{260}R$ was increased about 2.35 folds compared with the wild-type. This indicated that incorporation of two positively-charged arginine residues in the active site led to more interactions of these residues with the negatively charged-rich substrate and increased the phytase activity. In conclusion, highly active $T^{312}R/F^{260}R$ double mutant can be used as feed additive in feed industries.

Keywords: Phytic acid, Phytase, Targeted mutagenesis, Quickchange, Kinetics, Catalytic efficiency

INTRODUCTION

The second most abundant mineral in the body is phosphorus. Therefore, life is entirely dependent on phosphorus, and any deficiency of phosphorus or impaired absorption can reduce growth and lead to skeletal abnormalities [1,2]. Phytic acid (myo-inositol hexa-phosphate) is the main form of phosphate storage in plants. It has a molecular weight of 659.86 g mol^{-1} and a molecular formula of $C_6H_{18}O_{24}P_6$. Phytate refers to the salt of phytic acid and phytin to calcium/magnesium phytate [3]. Phytate phosphorus is phosphorus bound to phytic acid in cereals, legumes, oilseeds, and plant tissues, comprising about 50-80% of the total phosphorus of these plants [4,5]. In animals, phytic causes problems such as chelation of essential minerals [6,7], inhibition of digestive enzymes (alpha-amylase) [8], decease in the solubility of proteins in digestive tract [9], decrease in the bioavailability of phosphorus, and environmental contamination [10].

Phytase (myo-inositol hexakisphosphate phosphohydrolase) is a subset of phosphatase enzymes that hydrolyzes phytate through myoinositol phosphate intermediates and eliminates its anti-nutritional properties. The enzyme is found in a variety of living organisms such as plants, animals, and microorganisms (bacteria and fungi). However, microbial phytase are more promising candidates for commercial-scale production [11]. A large group of bacteria, filamentous fungi, and yeasts produce intracellular and extracellular phytases [7]. However, compared with fungal phytases, bacterial counterparts have higher optimum pH, higher pH stability, and higher substrate-specificity.

They are also resistant to the activity of proteases when they are introduced into the gastrointestinal tract of

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monogastric animals. Their molecular weight is varied from 37 to 55 kDa, and they are usually active at pH values between 4.5-8.5 and at a temperature of 25-70 °C. They are commonly used in the food industry to enhance food quality, as well as in bread preparation, improve plant growth, and reduce the level of phosphorus pollution in the environment [11]. The many benefits of phytases, including increased essential ions bioavailability and energy [12], improved protein digestion [13], and increased availability of phosphorus [14], led to a large increase in their industrial applications.

Among the several methods for enzyme stabilization, protein engineering by site-directed mutagenesis has been widely applied in the field of enzyme biotechnology. This type of mutagenesis is the process of making specific changes in the structure of a particular gene. Site-directed mutagenesis plays an essential role in molecular biology and facilitates research in many fields, such as regulatory elements in genes, protein-DNA interactions, proteins structures and enzymes activity and stability. This method is used to improve the activity and the stability of enzymes, as well as to alter the specificity and selective regulation of independent marker genes [15]. The site-directed mutagenesis by the Quick-Change^{TM} method is based on the PCR technique. The designed primers containing mutations are used to amplify double-stranded DNA template previously denatured using a powerful DNA polymerase to synthesize the mutant DNA. The use of *Dpn1* endonuclease to remove maternal methylated or semi-methylated sequences is a unique feature of this system. It should be noted that both primers must contain the desired mutation and the same start and endpoints on opposite strands of plasmid DNA. Both plasmid DNA strands are fully amplified linearly during PCR. As mentioned earlier, phytase is one of the most widely used industrial enzymes. In this regard, using docking programs and due to the negative charge of the substrate of the phytase, all possible mutation sites (residues) in the active site were replaced *in silico* to choose suitable sites for mutation and better and stronger bonding between the substrate and enzyme. Finally, by targeting mutations and replacing positively charged arginine with the active amino acids that constitute the active site, the likelihood of a stronger binding of the substrate to the enzyme is increased. It is hypothesized that these mutations could improve the enzyme activity. Therefore, the aim of this study was to investigate the effects of site-directed mutagenesis in the active site of phytase from *Yersinia intermedia on* the activity of the enzyme. In other words, the main aim of the study was to examine whether the incorporation of positively charged amino acids into the active site could increase the substrate-enzyme complex in the active site or not ($F^{260}R$ and $T^{312}R$ mutations) [16].

MATERIALS AND METHODS

Extraction of Vector Bearing Phytase Encoding Gene

In this study, phytase gene from *Yersinina intermedia* which had been cloned earlier in pET22b (+) vector (restriction enzymes: *EcoRI* and *HindIII*) was transformed into *E.coli* DH5α bacterial host and then after plasmid extraction, the amplified plasmid containing the phytase gene sequence was used as template for Quick change PCR [18].

Choosing Mutation Sites

First, the structure model of phytase from *Yersinia intermedia* was prepared and using SPDBV software, the modeled structure of phytase was analyzed. Then the structure model was compared to crystalized phytase structures available in the online database (https://www.rcsb.org/). In more detail, molecular docking study was performed using AutoDock Vina software to find mutated residues in the active site having higher binding energies for phytate. After choosing mutation sites, they were studied using DynaMut web server (http://biosig.unimelb.edu.au/dynamut/). In the web server, suitable mutation sites were chosen according to ΔG (kcal mol^{-1}) scores. In the next steps, by using WHAT IF and PIC webservers, the possibility of forming new ionic and hydrogen interactions among the mutated residues and other residues was calculated.

Site-directed Mutagenesis Primers Ddesign and Quick-Change PCR

Site-directed mutagenesis was performed by Quick-Change PCR using a pair of forward and reverse

Table 1. Characteristics of the Used Primers for Site-directed Mutagenesis in Phytase from Yersinia Intermedia

Primer name	Number of nucleotides	Sequence
F260P	35	5-CTGCACAATGCGCAACGTGATCTGATGGCAAAAAC-3
R260P	35	5-GTTTTTGCCATCAGATCACGTTGCGCATTGTGCAG-3
F312P	37	5-TTCCTGGGTGGTCATGATCGTAACATCGCCAATATTG-3
R312P	38	5-CAATATTGGCGATGTTACGATCATGACCACCCAGGAAC-3
T7 Promotor	20	5-TAATACGACTCACTATAGGG-3
T7 Terminator	19	5-GCTAGTTATTGCTCAGCGG-3

primers (Table 1) containing desired mutations (https://www.ncbi.nlm.nih.gov/). For mutagenesis, highly efficient DNA polymerase PrimeStar GXL DNA polymerase (Takara, Japan) was used and reagents, buffers and primers were added according to the manufacturer data sheet. Then, Quick-Change PCR mutagenesis was carried out in first and second round. In the first round, the reagents and buffers were mixed with forward and reverse primers in separate microtubes and placed in a thermocycler and incubated for five cycles as follows: 98 ºC for 20 s, 95 ºC for 45 s, 66 ºC for 45 s and 68 ºC for 45 s. Next, in the second round Quick-Change PCR, the first round microtubes containing each forward and revers primer was incubated in thermocycler again for 10 cycle as follows: 98 ºC for 20 s, 95 ºC for 45 s, 66 ºC for 45 s and 68 ºC for 45 s. Finally, the contents of each microtube containing each forward and reverse primer was mixed, and after the addition of extra volume of 0.3 µl of PrimeStar DNA polymerase (Takara, Japan), the microtubes were incubated for 15 cycles as follows: 95 ºC for 45 s, 66 ºC for 45 s and 68 ºC for 45 s.

Quick-Change PCR Product Transformation in Bacterial Host

Before the transfomtion of the Quick-Change PCR product into a bacterial host, it was treated with the *Dpn1* (Takara, Japan) to digest the maternally methylated strand in the plasmid. Then, the 2-5 µl of the product was transformed into susceptible *E. coli* (DH5α) and the heat shock method was applied for the transformation of the vector into competent cells. The microtubes containing competent bacterial cells and plasmid DNA were placed on ice to reach a temperature of 0 °C. Then, 5 µl of the desired plasmid was added to the competent cells and mixed gently with a sampler. After the incubation of competent cells in the presence of mutated vectors on in a water bath at 42 ° C for 90 seconds and then immediately transferred the microtubes to ice and left to cool down for 1-3 min. Afterward, 800 µl of antibiotic-free SOB (yeast extract 5 g l^{-1}, trypton 20 g l^{-1}, sodium chloride 0.5 g l^{-1}) (Merk, Germany) was mixed with 16 µl of 1M glucose (Merk, Germany) and 4 µl of 1 M $MgCl_2$ (Merk, Germany) in a microtube and allowed the cells to grow at 37 °C (130 rpm for 1 h). Finally, the microtube was centrifuged at 5000 rpm at 4 °C for 10 min, and the pellet was cultured on a solid 2XTY medium (yeast extract 10 g l^{-1}, trypton 16 g l^{-1}, sodium chloride 5 g l^{-1}, agar 16 g l^{-1}) (Merk, Germany). Then, after plasmid extraction using GeneAll plasmid extraction kit (GeneAll Biotechnology Co., Korea), the extracted mutated plasmids sequenced for the confirmation of mutagenesis [18].

Expression, Purification and Characterization of Wild-type and Mutated Phytases

In order to express and induce the wild-type and mutated phytases in the pre-culture phase, the plasmids extracted from the mutated bacterium were transformed into BL21 competent bacteria. Then, a single colony on solid Luria Broth (Merk, Germany) medium was removed and allowed to grow in a volume of 10 ml at 37 ºC at 130 rpm for 16 h. Afterwards, a 500 µl of pre-culture medium was added to a 100 ml of ampicillin (Sigma Aldrich, USA) medium and incubated at 130 rpm and 37 °C. When the bacterial culture medium OD reached 0.4-0.6, IPTG (Sigma Aldrich, USA) at a final concentration of 1 mM, was added and allowed to cool down at 30 ºC at 130 rpm for 6 h.

The medium was then centrifuged with the sample before induction at 4 °C at 6000 rpm for 15 min and cell precipitates were dissolved in 500 µl of a lysed buffer (50 mM tris, 300 mM sodium chloride, 10 mM imidiazol) (Merk, Germany), and were exposed to ultrasonication (power 80%, pulse 0.50). Finally, the samples were centrifuged at 15000 rpm at 4 °C for 30 min (https://www.novagen.com) [18].

Afterwards, for the purification of phytases, the supernatants containing wild-type or each of mutant phytases was added to nickel sepharose affinity chromatography column (Qiagene, USA). For purification, first, 8 ml of the resulting solution was added to the affinity chromatography column containing 2 ml of nickel sepharose (which had been equilibrated earlier with the lysis buffer) for 20 min at 4 °C. Then, using a washing buffer (50 mM tris, 300 mM sodium chloride, 20-40 mM imidiazol), nonspecific nickel sepharose-bound proteins having less than six histidine residues were removed. Afterwards, using an elution buffer (50 mM tris, 300 mM sodium chloride, 250 mM imidiazol), the recombinant wild-type and mutant phytases were eluted from the column and the protein fractions were collected. The concentration of purified phytases were measured by Bradford test and the quality of purification was investigated by SDS-PAGE [18,19]. The activity of the phytase enzyme was measured according to a method suggested by Sigma Aldrich Company (https://www.sigmaaldrich.com/content/dam/sigma-aldrich/docs/Sigma/Enzyme_Assay/phytaseec31326.pdf). Briefly, 0.05 µg of the phytase solution (wild-type or mutants) was added to a substrate solution (100 mM Magnesium sulfate, 6.82 mM phytic acid, sodium acetate 200 mM with pH: 5.15) and incubated at 55 °C. Then, at 0, 10, 20, 30, 40, 50, 60 min, aliquots of this mixture were added to 1 ml of TCA (10% w/v) to stop the enzyme activity. Then, Tausky-Shorr color reagent solution (TSCR) and distilled water were added to the solution and incubated for 5 min. The absorbance of the sample was read at a wavelength of 660 nm. Blank and Standard samples were prepared according to a method suggested by Sigma Aldrich company (https://www.sigmaaldrich.com/technical-documents/protocols/biology/enzymatic-assay-of-phytase.html)

RESULTS

Structural Modeling and *In Silico* Analysis of the Mutants

First, the three-dimensional structure of phytase from *Yersinia intermedia* was predicted in accordance with the enzyme structure of E. coli using I-TASSER server (https://zhanglab.ccmb.med.umich.edu/I-TASSER/. Mutation selection with minimum energy was performed using Chiron server (https://dokhlab.med.psu.edu/chiron/login.php). After modeling the structure of the mutants, three-dimensional structures were examined using SPBV-Viewer and Pymol software programs. The wild-type phytase and mutant forms of phytase were compared and analyzed. In order to choose the mutation sites, the substrate analog (D-MYO-INOSITOL-HEXASULPHATE) was docked with the active site of phytase from *Yersinia intermedia*. Then using DynaMut webserver ΔG values were predicted for the mutants. Altogether, docking study results and prediction of ΔΔG values by DynaMut webserver showed that F^{260} R and T^{312}R are appropriate mutation pairs for the stronger binding of the mutants to phytate (Fig. 1).

It was predicted that the binding energies of the substrate to the active site for the mutant phytases are increased and thus F^{260} R and T^{312}R mutants with higher binding energy to phytate were chosen (Fig. 2).

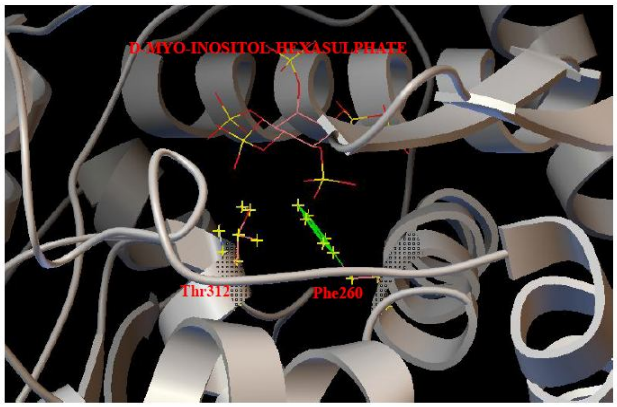
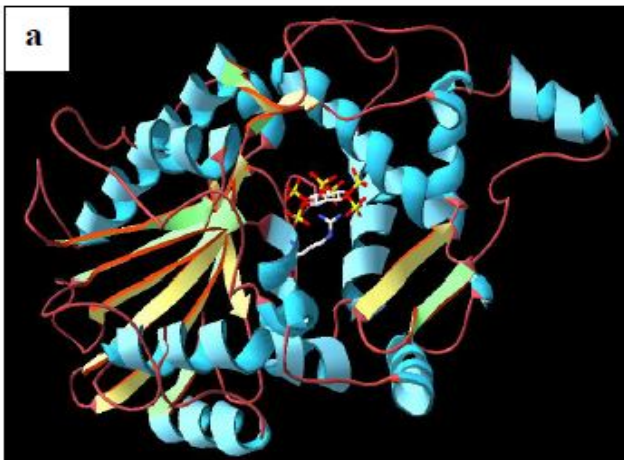


Fig. 1. Results of docking of mutated residues in the active site of phytase from *Yersina intermedia* and interactions among the mutated amino acidis and phytate.



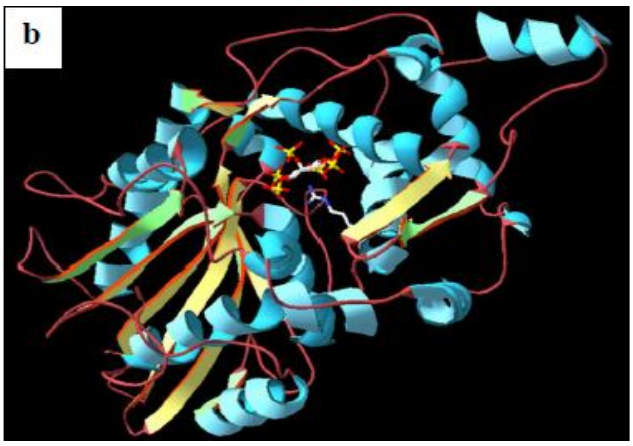


Fig. 2. Three-dimensional structural view of the phytase mutants (a) $T^{312}R$ mutant ; and (b) $F^{260}R$ mutant.

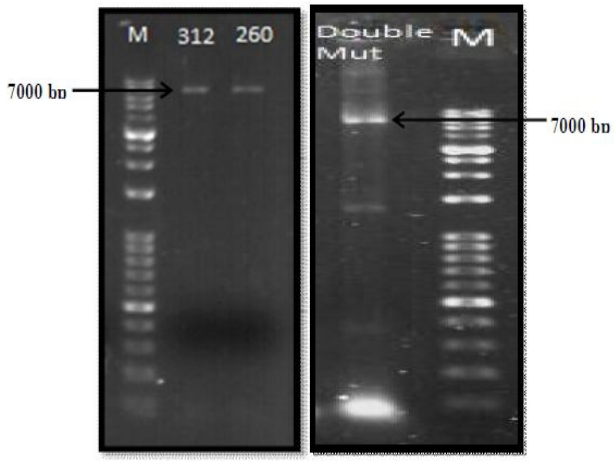


Fig. 3. The analysis of Quickchange PCR products for $T^{312}R$ and $F^{260}R$ single mutants and $F^{260}R/T^{312}R$ double mutant phytases (M: size marker) on 1% agarose gel electrophoresis.

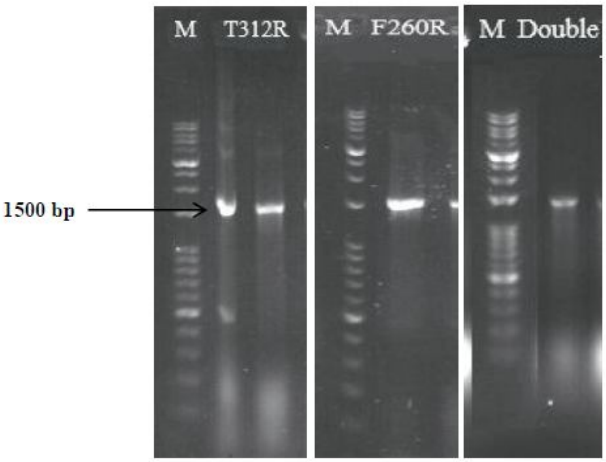


Fig. 4. PCR colony gel electrophoresis for $F^{260}R$, $T^{312}R$ and Double mutations (M: size marker).

Extraction of Wild-type and Mutated Plasmids

pET22b (+) vector containing the wild-type phytase was extracted using the commercial kit and mutated by Quick-Change PCR method. Then, the amplified linear plasmids containing $T^{312}R$ and $F^{260}R$ mutations, as well as $T^{312}R/F^{260}R$ mutation analyzed on agarose gel electrophoresis (Fig. 3). The band size of the mutant plasmids which had been amplified by site-directed mutagenesis was estimated to be approximately 7000 bp.

Transformation of Mutant Plasmids Encoding Phytase in Bacterial Hosts, Expression and Purification of the Enzymes

Quick-change PCR product was treated with the *Dpn1* to remove the maternally methylated strands. Then, the newly synthesized mutated strands were transformed into the *E. coli* DH5α bacteria. As shown in Fig. 4, grown colonies were randomly examined by PCR using a pair of forward and revers T7 primers (Table 1). According to Fig. 4, the amplicon length of colony PCR products was When the sequencing results of mutant phytases were estimated to be approximately 1500 bp.

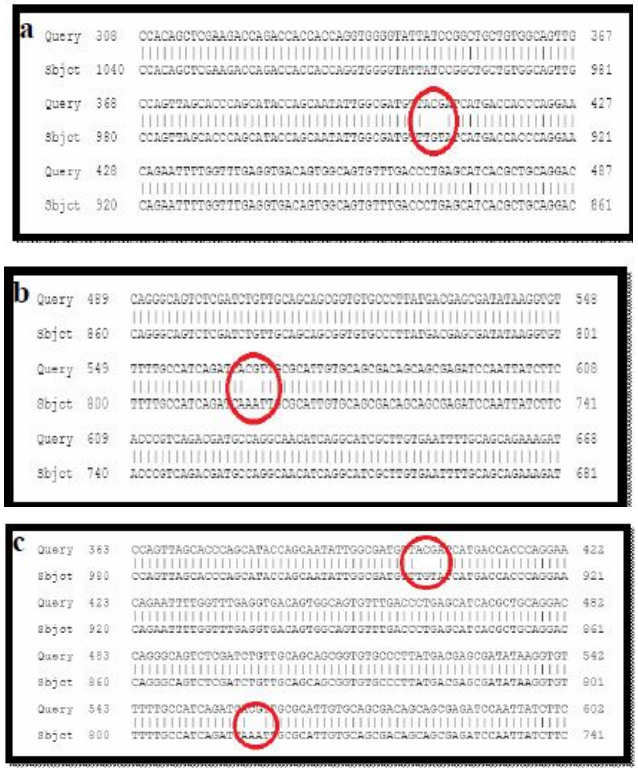


Fig. 5. Nucleotide BLAST search results for mutant phytases (a) $T^{312}R$ single mutant; (b) $F^{260}R$ single mutant; and (c) $F^{260}R$ / $T^{312}R$ double mutant phytase.

received from Macrogen inc., the sequence of each mutant phytases was aligned with that of the wild-type *via* the NCBI BLAST tool (https://blast.ncbi.nlm.nih.gov/Blast.cgi) as shown in Fig. 5 and mutagenesis was confirmed.

After expression and purification of wild-type and mutant phytases, to evaluate the quality of purified enzymes, they were analyzed on SDS-PAGE as depicted in Fig. 6 and the molecular weight of phytases was approximately 42 kD. Then, the activities of wild-type and mutant phtytase were measured in the presence of phytate, as substrate, using a phosphorus standard linear plot (Fig. 7).

DISCUSSION

As reported earlier phytase produced from *Yersinia intermedia* has shown the highest activity among wild-type

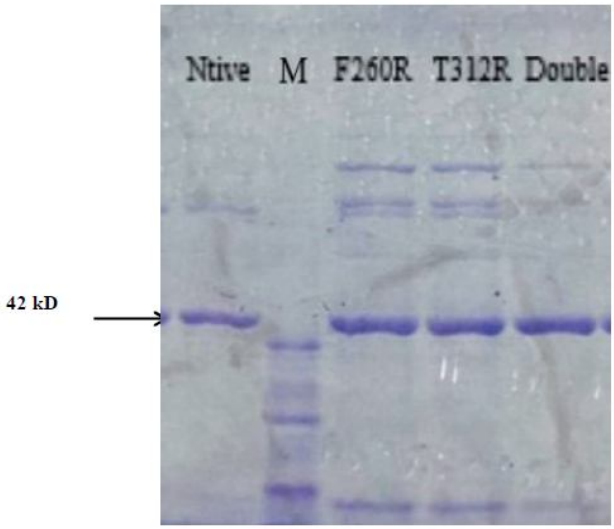


Fig. 6 . Analysis of purified wild-type , $F^{260}R$ single mutant , $T^{312}R$ single mutant and $F^{260}R/T^{312}R$ double mutant phytases on 12% SDS-PAGE (M: protein size marker).

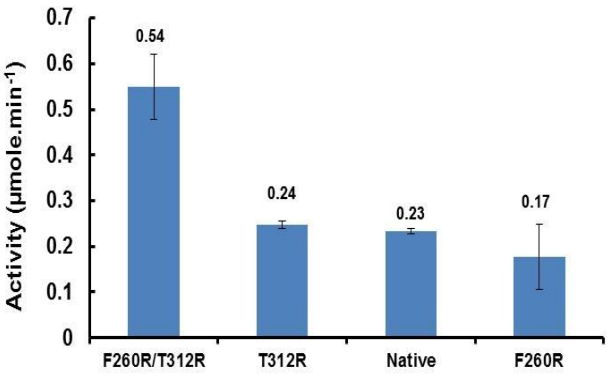


Fig. 7. Measurement of the activity of wild-type and mutant phytases from *Yersinia intermedia*. Briefly, 0.05 µg of the phytase solution (wild-type or mutants) was added to 20 ml of the substrate solution (100 mM magnesium sulfate, 6.82 mM phytic acid, 200 mM sodium acetate (pH 5.15) and incubated at 55 °C. Then, at 0, 10, 20, 30, 40, 50, 60 min, 2 ml of this mixture was added to 1 ml of TCA (10% w/v) to stop the enzyme activity. Followingly, 5 ml of Tausky-Shorr color reagent solution (TSCR) and 2 ml of distilled water were added to the solution and incubated for 5 min. Finally, the absorbance of the sample was read at a wavelength of 660 nm.

bacterial phytases [20]. Based on the results and according to the purpose of the study, phytase was actively expressed in *E.coli* BL21 bacterial host. According to the results, mutagenesis in the active site of phytase from *Yersinia intermedia* led to the improvement of the binding energy of the substrate and increase in the activity of the double mutant phytase. Based on the results of myo-inositol analog docking as substrate with the active site of phytase, several mutation sites (residues) were chosen in a way which have a better and stronger binding between the substrate and enzyme. However, according to the predicted ΔG values of structural stability for mutant phytases, $T^{312}R$ and $F^{260}R$ mutations were chosen. It is worthy to note that the phytase gene used in this study has been codon optimized previously [20]. Comparison among the values of mutant enzyme activities indicates that the enzyme activities of single mutant phytases didn't change significantly *versus* the wild-type. However, the activity of double mutant phytase increased by 2.35 fold compared with that of the wild-type. No significant change was observed in terms of pH and optimum temperature and optimum pH for the mutants and even temperature remain 5.15 and 55 °C. Possibly, the single mutants have not led to substantial alterations in the conformation and the binding capacity of phytase active site. Therefore, it can be hypothesized the active site's microenvironment remained intact and thus the pKa values of amino acids at this site also remained unchanged. However, the condition for double mutant phytase is different. According to the results, double mutations in the active site of the phytase could have positive synergistic effects such that the activity of the enzyme significantly has been increased. It can imagined that double mutations might have resulted in increase in the flexibility of active site which in turn increases the rate of the entry of phytate to active site and also the rate of release of the enzyme's products. These mutations increased the enzyme activity, while optimum temperature and pH values remained unchanged. In agreement with our study, Fu and colleagues in 2011 mutated phytase from *Yersinia enterocolitica* and according to their results, mutagenesis caused a significant decrease in the activity of the mutant phytases compared with the wild-type enzyme. They have demonstrated that mutation with adverse effect on the enzyme activity is due to fundamental structural changes or due to changes in the pKa values of the residues located in the active site of the enzyme [20]. In contrast to our results, Liao and colleagues in 2013 showed that a double mutant phytase from *Aspergillus niger* led to an increase in the enzyme's thermal stability compared the wild type [21]. Based on a research conducted by Chen and coworkers in 2015, multiple mutations in the active site of phytase from *Bacillus subtilis* caused a remarkable increase in enzyme's activity [22]. Next year, Shivange and coworkers in 2014 reported that mutagenesis in the enzyme's active site from *Yersinia mollaretii* increased enzyme's activity versus the wild-type [23]. In the same year, According to a study conducted by Zhang and colleagues in 2016, the optimum pH value of phytase from E.coli decreased after the substitution of Lys by Glu in the enzyme's active site [24]. In another study, Zhou et al. in 2019 demonstrated that mutagenesis in the active site of phytase from *Aspergillus neoniger* led to an increase in the enzyme's optimum pH value [25] (Table 2). Considering above-mentioned discussion, it can be concluded from the results of the current study that the mutated residues (Phe 260 and Thr 312) play a key role in phytase active site. Mutated phytase with improved activity provides extraordinary conditions for feed and food industries.

CONCLUSION AND FUTURE PERSPECTIVE

The addition of highly active phytase to feed and food industries increase the bioavailibity of phosphorus and divalent cations while decrease the soil and water pollution. High active double mutant $F^{260}R/T312R$ phytase can hydrolyze phytae in bread and paper industries. Using this high active mutant phytase less amount of enzyme is needed for the hydrolysis of phytate. Altogether, the double mutant $F^{260}R/T^{312}R$ phytase has potential to be used in food and feed industries to increase the nutritional value of food and feed. Besides, the double mutant phytase can be used in transgenic monogastric animals to increase the bioavailability of phosphorus and essential divalent cations and also to prevent environmental pollution. Furthermore, it can be used in and transgenic plants to produce phosphorus-rich cereals, seeds and legumes [27].

Table 2. Site-directed Mutagenesis in the Active Site of Phytases from Different Microbial Species

	Species	Mutation site	Mutation output	Ref.
1	*Aspergillus niger N25*	Loops near active site	35% Enhancement in thermal stability	[21]
2	*Bacillus subtilis*	Loops close to active site	Increase in catalytic efficiency and specific activity	[22]
3	*Yersinia mollaretii*	Next to the active site loop, surface, α-domain, α/B domain	54% Enhancement in residual activity	[23]
4	*Escherichia coli*	Surface of pocket edge	-0.5 Decrease of pH optimum	[24]
5	*Aspergillus neoniger*	Catalytic motif	-0.2 Decrease in pH optimum	[25]
6	*Yersinia intermedia*	Active site	Unchanged	-

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