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SITE-DIRECTED MUTAGENESIS OF THE BACTERIAL PHYTASE GENE COMPATIBLE FOR CHICKEN CODON PREFERENCES

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ABSTRACT

Phytases are among a special group of phosphatase enzymes that catalyze the stepwise hydrolysis of phytic acid and release a usable form of inorganic phosphorus. Currently, in order to increase the absorption of dietary phosphorus and decreasing the phosphorus pollution in the environment, phytase could be supplemented to the diet of monogastric animals including pig, poultry and fish. Commercially available exogenous phytases are commonly derived from either fungi, yeasts or bacteria, Nevertheless, *E. coli* bacteria is one of the main sources of phytase expression, that produces a type of phytase which is resistant to pepsin hydrolysis in the stomach of most animals, and also has a high specific activity for hydrolzying phytic acid. The aim of this study was the isolation of the bacterial *phytase* gene and optimizing its encoding sequences for efficient expression in chicken. At the first step, through specific primers, cloning of this gene was achieved by insertion of the DNA fragment encompassing bacterial *phytase* gene to *pTG19* vector. This fragment was then inserted into the pIRES-hrGFP-vector. This recombinant vector is suitable for further analyses to investigate the expression of the phytase gene along with the EGFP transgene.

Key words: Phytase, *E. coli*, codon bias, IRES vector, chicken.

INTRODUCTION

Defecated phosphorous from animal's digestive system is the main source of phosphorous pollution in both water and soil ecosystems (Jongbloed and Leins, 1998). Phosphate excess in water sources can induce eutrophication and subsequent hypoxia influx and nitrous oxide formation. Afterwards, these lead to the death of various fish species which are the main component of human food basket in the coastal areas. On the other hand, increase in the green house gases from the eutrophication process is another environmental concern of the excessively excreted phosphorous (Golovan *et al.*, 2001a). Because of a lack of proper phytate digestive ability, monogastric farm animals, which are domesticated to meet human foods, defecate the phytate of beans (and grain) in the environment. Moreover, this monogastric limitation decreases the digestible energy (Persia *et al.*, 2002) while increasing the calcium (Nelson *et al.*, 1968) and protein (Cowieson *et al.*, 2004) requirements. Broilers and layers are commercially produced around the globe. Essential nutrient requirements for broiler chicks are met using either inorganic phosphorous sources which are not fully utilized or phytase enzyme supplementation as a feed additive (Jongbloed and Kemme, 1990). The required phosphorous and calcium is in a higher shortage in the layer diet (Persia *et al.*, 2013). Therefore, providing an appropriate source of available phosphorous is imperative in the chicken industry to maximize the production efficiency and minimize the phosphorous remnants in chicken feces.

Phytate has direct interaction with the gastro-intestinal tract through mediating the immune response and increasing gastro-intestinal maintenance energy and it collectively decreases poultry performance (Persia *et al*., 2003). Phytase enzyme supplementation noticeably improved the chick performance (Murugesan *et al.*, 2014), available crude protein, metabolizable energy, feed conversion rate, and it decreased the mortality rate (Persia *et al*,. 2002; Lumpkins *et al.*, 2009; Murugesan *et al.*, 2014). At high level of consumption, phytase source was not significantly effective on the chicken performance (Persia and Saylor, 2006). The mechanism for higher performance at higher phytase level has been proposed to be through decreasing immune response, mucin production and endogenous loss (Persia *et al.*, 2013).

However, inclusion of mineral phosphorous may interact with the adsorption of calcium and other minerals. Phytase enzyme also might be degraded or denatured during the pellet formation or feed process. Therefore, devising an environmentally friendly approach for efficiently utilizing the phytate's phosphorous with the least interaction with digestibility of the other minerals would be a great milestone in the chicken industry. Herein, we propose production of the phytase enzyme in the chicken gut per se by making transgenic chickens carrying an efficient bacterial phytase gene. This strategy has been successfully accomplished in pigs by production of the Yorkshire Enviropigs^{TM} which

secrete the *E. coli* phytase transgene in their salivary glands (Forsberg *et al.*, 2012). Therefore, the aim of this study was to clone the bacterial phytase gene in an appropriate expression vector applicable for chicken transgenesis.

MATERIALS AND METHODS

Bioinformatics studies: Nucleotide sequences of bacterial (*E.coli*) *Phytase* gene (NC_007779.1) was acquired through the NCBI data bank. The primer pair sequences for trivial phytase amplification were as follows: Sense oligonucleotide: 5'-CTCGAGATGAAAGCGATCTTAATCCCAT-3' and antisense oligonucleotide: 5'-GGGAATTCATTACAAACTGCACGCCG-3' (Golovan *et al*, 2000). Primers were ordered through Alpha DNA Company (Canada).

Cloning and mutational insertion: *Escherichia coli* bacterial cells, K12 strain, were purchased from the Pasteur Institute, (Tehran, Iran). DNA extraction was performed from bacterial cells using conventional method, phenol- chloroform procedure (Green and Sambrook, 2012). The quantity and quality of extracted DNA was assessed by a Nano drop spectrophotometer (Nanodrop, U.K.) and electrophoresis on agarose gel 1%. PCR reaction to amplify the coding sequence of *Phytase* gene was carried out with the aforementioned primers. A conventional PCR approach was performed using Ex*Taq* DNA polymerase (TaKaRa, Japan) to amplify this fragments was as follows: Initial denaturation at 94ºC for 5 min followed by 35 repetitive cycles including 94ºC for 35 sec as denaturation step, 58 ºC for 40 sec as annealing step, 72 ºC for 45 sec as extension step. At last, PCR was terminated after final extension at 72 ºC for 5 min. The amplified fragment (1299bp) was electrophoresed on 1% agarose gel. Then Phytase CDS was extracted from the gel using DNA extraction kit (Thermo Scientific, USA). This fragment was inserted into the pTG19-T PCR cloning vector (SinaClon, Iran) during T/A cloning procedure using DNA Ligation Kit, Mighty Mix (TaKaRa, Japan) as described in manufacturer manual (Figure 1). Recombinant plasmid (pTG19-T/ *Phytase* CDS) was sent for sequencing (Bioneer, Korea) with universal primers (M13 forward and reverse primers) to ensure the accurate amplification of *Phytase* CDS without undesired mutation(s).

At the next step, according to the most codon usages in chicken (Rao *et al*. 2011), the nucleotides of codon numbers 2, 3, 4, 5, 6, 7, 9 and 425, 426, 427, 428, 429, 430, 431, 432 were improved (Table1). To introduce new optimized codon, a directional mutagenesis PCR strategy was implemented with a couple of primers (Table 2) which were ordered through Alpha DNA Company. To achieve the amplification, touch-down approach was carried out with the ordered primers of Table 2. PCR was performed in an Eppendorf thermal cycler (6321XQ401064, Eppendorf, Germany) using pTG19-T/ *Phytase* CDS as a template with Ex*Taq* DNA polymerase. Touch-down PCR was carried out with one step of initial denaturation at 95ºC for 5 min followed by twenty repetitive cycles, including 94ºC for 45 sec, a variable annealing step and an elongation step at 72ºC for 45 sec. The variable annealing step was started at a temperature of 70ºC which decreased by 0.4ºC/cycle to reach a temperature of 62 ºC for 30 sec. Subsequently, additional amplification was continued for 10 cycles with a constant annealing temperature of 60ºC for 30 sec. PCR was terminated after final elongation step at 72ºC for 10 min. The amplified fragments (*Eco*R1- *Phytase* CDS-*Xho*I) were extracted from the gel as previously described. *Eco*R1- *Phytase* CDS-*Xho*I was treated with the respective restriction enzymes to produce sticky ends. At the next step, treated fragment was inserted into the same sites in MCS of pIRES-hrGFP-1a vector (Agilent Technologies, USA) through a ligation procedure with DNA Ligation Kit, Mighty Mix (Figure 2).

RESULTS AND DISCUSSION

In this study, we used *E. coli* phytase gene from B12 strain as this phytase's protein efficacy has been verified in the digestive system of mice and pigs (Golovan *et al.* 2001a; Golovan *et al.* 2001b). Nevertheless, a recent study has shown a new phytase source from *Kelebsiella pneumonia* 9-3B associated with enhanced resistance to heat and pH variations (Escobin-Mopera *et al.*, 2012). However, the expression pattern and protein functionality of this new resource still remain to be verified in eukaryotic cells.

As depicted in the Figure 2A, Phytase CDS was amplified appropriately. The electrophoresis based on the agarose gel resulted in the observation of our desired bands. This amplified band was then extracted from the gel and inserted into the pTG19-vector as described in materials and methods (a schematic presentation is provided in Figure 3A). At the next step, a set of mutations was induced according to the codon optimized approach for efficient transfection into the chicken genome with the specific primers and PCR strategy depicted in Figure 3B. The optimized amplified fragment encompassing the phytase CDS was accompanied with XhoI and EcoRI sites at both ends (Figure 4). Afterwards, this fragment was digested by the above-mentioned restriction enzymes to produce a newly site-directed mutated fragment (EcoR1- Modified Phytase CDS-XhoI) for further insertion in to the same site in the pIRES-hrGFP-1a vector (Figure 4). The recombinant vector was termed pIRES-hrGFP-1a/ Chicken Phytase.

E. coli K12 cells were transformed with this recombinant vector and several bacterial colonies were

picked for evaluation of colony insert check. Implementation of the colony insert-check assay indicated positive colonies propagating our recombinant vector of interest (Figure 5). Among these positive colonies, three colonies were randomly selected for further amplification steps followed by DNA sequencing. The sequencing specified that the phytase amplicons, pIRES-hrGFP-1a/Chicken Phytase, which was properly codon-optimized, is replicated in the assessed colonies. This plasmid contains codon- optimized sequences which are appropriate for expression in the chicken. Domestication of exogenous genes via contextualizing the codon sequences for each specific host animal has been an accepted modification for overcoming cellular defense mechanism and subsequently the gene sequestration and silencing phenomenon. This approach has been successfully carried out in transgenic mice and pigs which carried the partially domesticated K12 *E. coli* phytase as an exogenous DNA source (Golovan *et al.*, 2001a; Golovan *et al.*, 2001b; Yin *et al.* 2006). In the current study, we optimized eight amino acid codons at the early part of the transgene and another seven amino acid codons at the late part of the phytase open reading frame based on Rao *et al*. (2011). The number of mutated codons in this study was higher than for the mammalian contextualized phytase (Golovan *et al.* 2001a). Moreover, application of internal ribosomal entry site (IRES) system for dual expression of two transgenes, which are transcribed under a unique promoter and using an IRES as a spacer and ribosomal binding site, has been widely documented to investigate the cellular transfection efficiency by evaluation of enhanced green fluorescent protein (EGFP). In the next part, the project is aimed to evaluate the expression efficiency of the modified construct under various tissue specific promoters for final usage in chicken transgenesis.

Even for coprophage state of mice which recycle phytate, transgenic mice carrying the phytase transgene under R15 promoter showed 11% reduction in fecal dry matter phosphorous concentration compared to the control group (Golovan *et al.* 2000). Similar results have been achieved using the parotid secretory protein as the promoter and the bovine/swine growth hormone polyadenylation signal as the termination sequence (Yin *et al.* 2006). Concluding annotations from our current study as well as the so far produced transgenic mammals carrying the phytase gene (Forsberg *et al.* 2012) suggested that mammalian transgenesis aiming for gastrointestinal expression of phytase is a long term plausible and cost-effective approach compared to supplementing the phytase enzyme which already underwent several processing steps for the endpoint product.

The current results were achieved from the first part of a long-term project aiming to make transgenic chicks efficiently expressing the *E. coli* phytase in their digestive system. At the next steps of this project, we are going to evaluate the expression efficiency of this codon-optimized gene which is currently under the human cytomegalo virus (hCMV) promoter, through pIRES-EGFP vector, in the chicken embryo fibroblast cells. Finally, the construct will be evaluated under a set of tissue specific promoters and their expression efficiency will be evaluated in the transgenic chickens carrying the constructs.

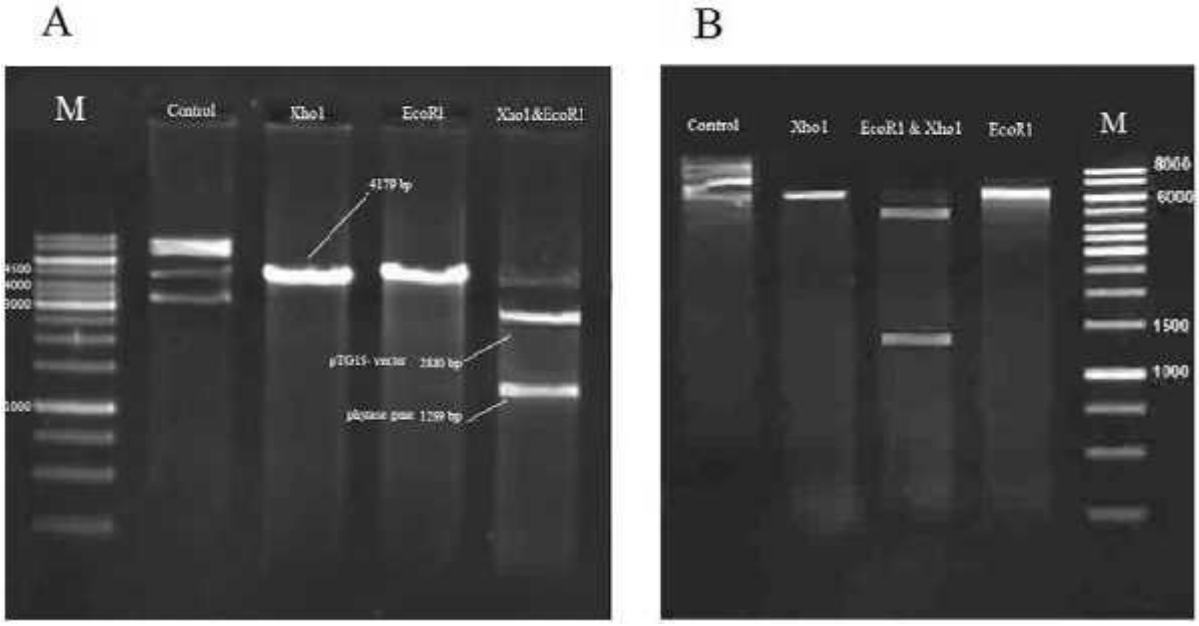

Figure 1. Schematic presentation of primers for amplification of *E. coli* phytase (A1 and A2). Another set of primers in which contextualized site-directed mutagenesis was carried out for chicken codons is shown in B1 and B2.

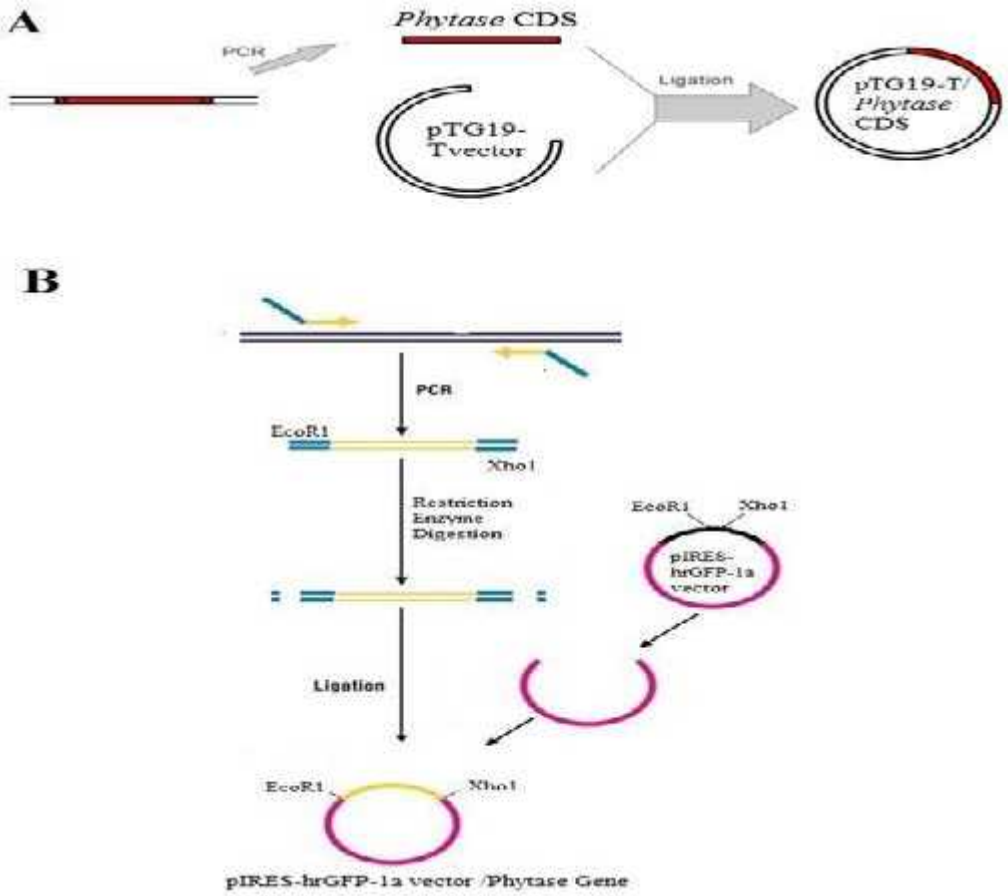

Figure2. PCR amplicons from *E. coli* phytase primers (A) and the modified amplicons for chicken expression.

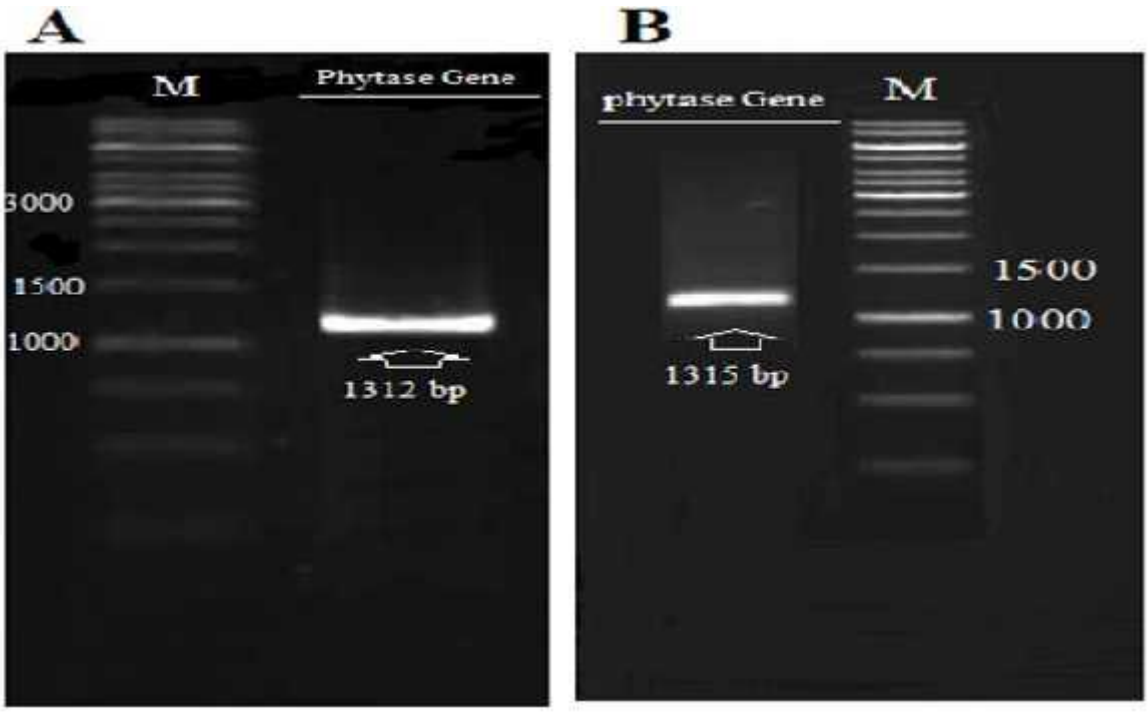

Figure3. Schematic presentation of *E.coli* phytase gene cloning in pTA vector (A) and pIRES-EGFP vector (b).

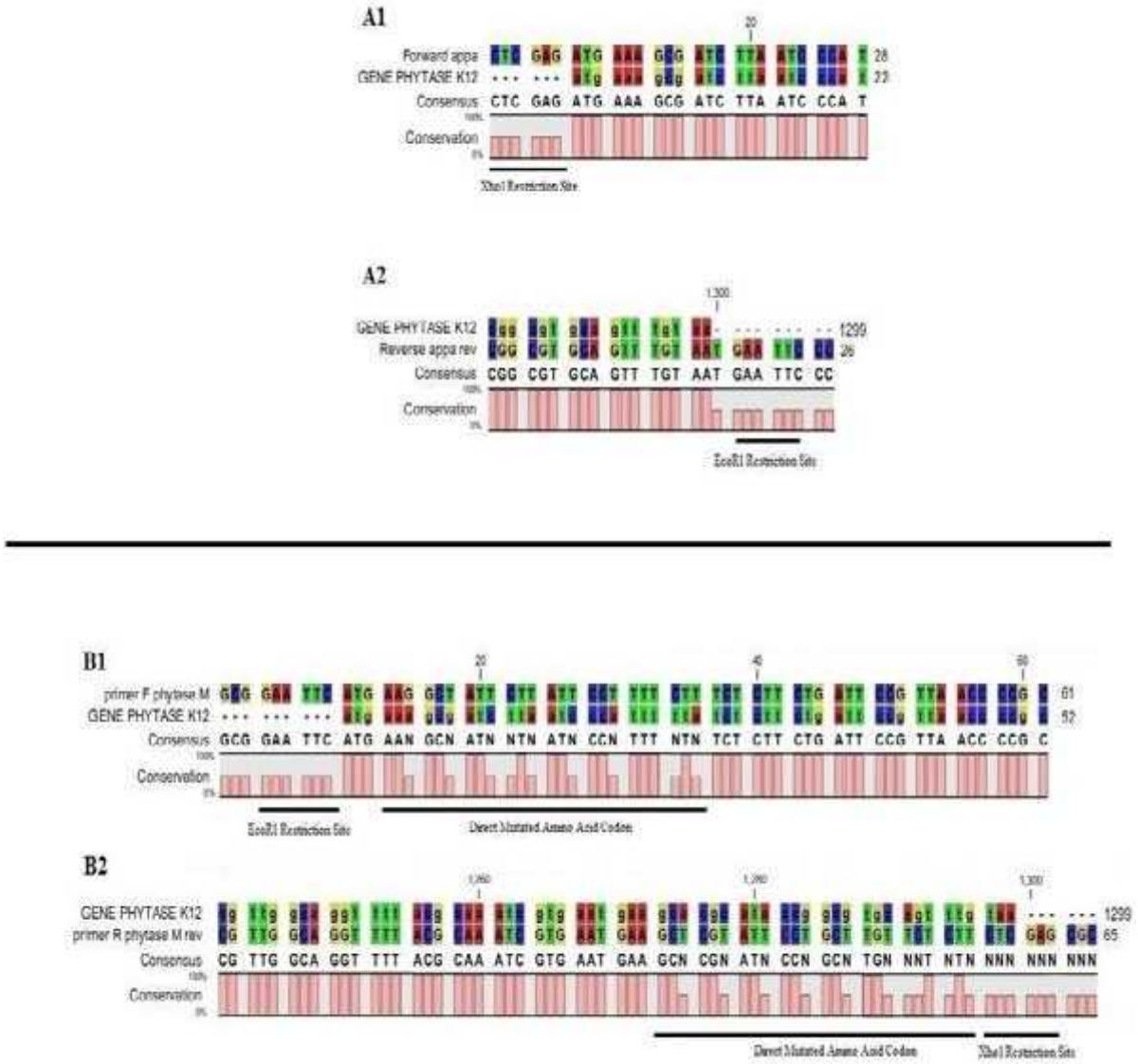

Figure4. Restriction enzyme digestion of cloned phytase in pTA vector (A) and pIRES-EGFP vector (B).

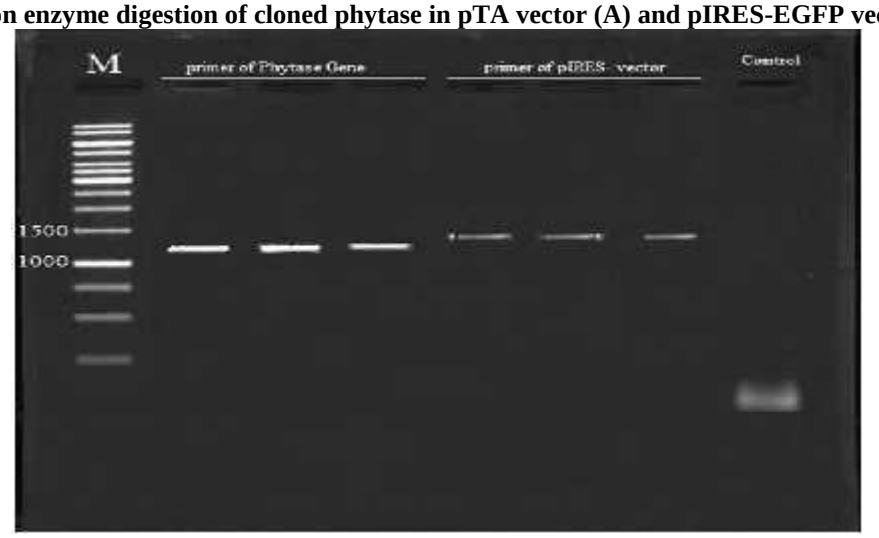

Figure 5. Colony-PCR results based on modified *E.coli* phytase primers (A) and primers outside of the amplicon in pIRES-EGFP vector (B).

Table 1. Optimized codons of E. coli phytase gene based on chicken genome codon bias.

Modified codon number	Modified nucleotide number	Phytase E. *coli* codon	Preferred *Gallus Gallus* codon
2	6	AAA	AAG
3	9	GCG	GCT
4	12	ATC	ATT
5	13,15	TTA	CTT
6	18	ATC	ATT
7	21	CCA	CCT
9	25,27	TTA	CTT
425	1275	GCA	GCT
426	1278	CGC	CGT
427	1281	ATA	ATT
428	1284	CCG	CCT
429	1287	GCG	GCT
430	1290	TGC	TGT
431	1291,1292	AGT	TCT
432	1294,1296	TTG	CTT

Table 2. List of primer pairs used for directional mutagenesis by PCR method

Name	Sequence
Chick_Phyt_F	5'-GCG GAATTC ATG AAG GCT ATT CTT ATT CCT TTT CTT
	TCTCTTCTGATTCCGTTAACCCCGC-3'
	EcoR1 M K A I L I F
Chick_Phyt_R	5'-GCG CTCGAG AAG AGA ACA AGC AGG AAT ACG AGC
	TTCATTCACGATTTGCGTAAAACCTGCCAACG-3'
	Xho1 L S C A P I R A

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