Construction of a Probiotic Lactic Acid Bacterium that Expresses Acid-Resistant Phytase Enzyme

R. Majidzadeh Heravi^{1*}, M. Sankian², H. Kermanshahi¹, M. R. Nassiri³, A. Heravi Moussavi¹, L. Roozbeh Nasiraii⁴, and A. R. Varasteh⁵

ABSTRACT

The use of genetically engineered probiotics to express specific enzymes has been the subject of considerable attention in poultry industry because of increased nutrient availability and reduced cost of enzyme supplementation. Phytase enzyme is commonly added to poultry feed to improve digestibility and availability of phosphorus from plant sources. To construct a probiotic with potential of phytate degradation, *phytase* gene (appA) from *E. coli* was cloned and transformed into two probiotic bacteria *Lactobacillus salivarius* and *Lactococcus lactis*. The results showed plasmid instability, unable to express the gene. The expression of *appA* gene in *L. lactis* was analyzed by detecting specific RNA and zymography assay. Phytase enzyme was isolated from cellular extracts of recombinant *L. lactis*, showing a 46 kDa band upon the SDS-PAGE analysis. Zymogram also confirmed the phytase activity of the 46 kDa band corresponding to the enzyme. An enzyme activity of 4.9 U mL⁻¹ was obtained in cell extracts of *L. lactis*. The growth of native and recombinant *L. lactis* was similar in the presence of two concentrations of ox bile.

Keywords: Lactobacillus salivarus, Lactococcus lactis, Recombinant, Phytase, Poultry.

INTRODUCTION

Phosphorus, as an essential mineral, is usually not bioavailable to non-ruminant animals in phytate form due to the absence of phytase enzyme in the digestive tract, which is required to separate phosphorus from the phytate molecule. Phytase enzyme increases the bioavailability of phosphorus in animal feeds by cleaving Phosphate (PO_4) band from plant phytate (myo-inositol hexakisdihydrogen phosphate) (Lei and Stahl, 2001). A phytase enzyme similar to phosphorus supplement has been shown to be efficacious for the improvement of bone mineralization, the growth performance, and productive trait of mono-stomach animals (Veum *et al.*, 2006; Augspurger *et al.*, 2007). Phytate represents about 60-90% of total phosphorus in cereal grains and oilseeds (Veum *et al.*, 2006). Given that the existence of residual phosphorus in fecal material contaminates environment and water resources, it is highly important to increase the absorption of phosphate from digestive tract of mono-stomach animals.

The commercial phytase are often obtained from fungi or bacterial sources. So far, *Aspergillus niger* phytase, which exhibits high

¹ Department of Animal Science, Faculty of Agriculture, Ferdowsi University of Mashhad, Mashhad, Islamic Republic of Iran.

*Corresponding author; e-mail: rmajidzadeh@um.ac.ir

² Immuno-Biochemistry Laboratory, Immunology Research Center, Medical School, Mashhad University of Medical Sciences, Mashhad, Islamic Republic of Iran.

The Research Institute of Biotechnology, Ferdowsi University of Mashhad, Mashhad, Islamic Republic of Iran.

⁴ Department of Food Science, Nour Branch, Islamic Azad University, Nour, Islamic Republic of Iran.

⁵ Immuno-Biochemistry Laboratory, Allergy Research Center, Medical School, Mashhad University of Medical Sciences, Mashhad, Islamic Republic of Iran.

activity, has been successfully expressed in Pichia pastoris (Xiong et al., 2005) and Saccharomyces cerevisiae (Han et al., 1999). The Escherichia coli phytase gene appA has also been a suitable target for expression in Schizosacchromyces pombe, S. cerevisiae and P. pastoris (Lee et al., 2005). It has been demonstrated that E. coli phytase is resistant to pepsin enzyme with an optimum activity resembling the physiological pH in the stomach of chickens and pigs (Lei and Stahl, 2001). It provides an excellent condition for phytate digestion as the molecule of phytate is soluble in the acidic condition of stomach. Also it has been revealed that stomach digesta can efficiently inactivate the enzymes, except for E. coli phytase which displayed a residual activity of 93% after 60 minutes incubation at 40°C (Igbasan et al., 2000).

Lactic acid bacteria are a type of probiotic bacteria with poor phytase activity caused by pressures (Kerovuo evolutionary and Tynkkynen, 2000). There are a few reports about the cloning of phytase gene in a Lactic acid bacterium. In a study, the phytase gene Aspergillus ficuum (appA) from was successfully expressed in Lactobacillus casei (Zuo et al., 2010). Also, a weak expression of Bacillus subtilis phyC gene was obtained in L.

plantarum (Kerovuo and Tynkkynen, 2000). To date, there has been a paucity of reports on transferring *E. coli phytase* gene into *lactobacilli* and *lactococcus* genus.

The present study aimed at cloning and expression of *E. coli phytase* gene (*appA*) in a species of *L. salivarus* isolated from gastrointestinal tract of healthy broiler chickens and in *Lactococcus lactis* subsp. *Cremoris* MG1363 (Mierau and Kleerebezem, 2005) as a probiotic species. Probiotic criteria of *L. salivarus* have already been examined and proved in previous *in vitro* studies (Majidzadeh Heravi *et al.*, 2011).The aim of this study was to construct a probiotic with phytate degradation potential.

MATERIALS AND METHODS

Bacterial Strains, Plasmids, and Media

The bacterial strains and plasmids used in this study are listed in Table 1. *L.salivarus* and *E.coli* were grown in MRS (De Man Regosa and Sharp) and Luria-Bertani (LB) broth at 37°C, respectively. *L. lactis* was cultured in M17 broth at 30°C. Solid media were provided by adding 1.5% agar to each

Table 1. specification of Bacteria and plasmids used in this study.

Bacterium or plasmid	Relevant feature (s)	Source and references		
Bacteria				
Lactococcus lactis subsp.	pepN::nisRnisK	(Mierau and Kleerebezem,		
Cremoris MG1363 (NZ9000)		2005)		
Lactococcus lactis pBu003	pepN::nisRnisK; carrying pBu003	This study		
Lactococcus lactis pBuappA	pepN::nisRnisK; carrying pBuappA	This study		
Lactobacillus salivarous	Isolated from gastrointestinal tract of	(Majidzadeh Heravi <i>et al.</i> ,		
E. coli MC1061	broller chickens araD139, Δ(ara, leu)7697, ΔlacX74 galU-, galK-, hsr-, hsm+, strA	MoBiTec GmbH, Germany		
Plasmids				
pNZ3004	Cm ^r Em ^r ; <i>E. coli-lactobacillus</i> shuttle vector; 4.9 kb	e (van Rooijen <i>et al.</i> , 1992)		
pBu003	Cm ^r Em ^r ; <i>E. coli-lactobacillus</i> shuttle	e This study		
pBu <i>appA</i>	Cm ^r Em ^r ; pBu003 containing <i>E. coli</i> <i>appA</i> gene, 6.3 kb	i This study		

medium. To achieve selective media, the media were supplemented with 5, 10 and 400 μ g mL⁻¹ of erythromycin (Sigma-Aldrich, Missouri 63103 USA) for *L. salivarus*, *L. lactis*, and *E. coli*, respectively.

DNA and Plasmid Isolation

DNA was isolated from *E. coli* BL21 DE3 for amplification of *appA* using Chachaty and Saulnier method (2000). Plasmid DNA was extracted from *E. coli* MC1061 based on alkaline lysis method (Sambrook and Russell, 2001). Restriction enzymes and T4 ligase were purchased from Fermentas Corporation (Fermentas GMBH, Germany).

Plasmid Modification

pNZ3004 contains only two restriction sites for *Sal*I and *Pst*I restriction enzymes in the cloning site and one of these i.e. *SalI*, is also present in the *phytase* gene (appA). A 58 bp fragment containing five restriction sites was designed and inserted in *SalI-Pst*I sites. The modified plasmid was designated as pBU003.

Construction of Phytase Expression Plasmid

The coding sequence of *appA* gene (AM946981.2) from *E. coli* BL21 was amplified with the forward primer E1 (5'-ATCCTT<u>CTCGAG</u>TCTGCATTCGCTCAG AGTGAG-3') and the reverse primer K1 (5'-GGTTT<u>GCGGCCGC</u>TTACAAACTGCAC GCCGGT-3'). These primers were designed to add an *Xho*I site (underlined) to the 5' end and a *Not*I site (underlined) to the 3' end of the PCR product. The PCR fragments encoding phytase digested by *Xho*I and *Not*I enzymes were inserted into an *XhoI-Not*I digested pBU003 to construct pBU*appA* (Figure 1).

Transformation of Plasmid DNA

Competent *E. coli* MC1061 were prepared and transformed by the standard method (Sambrook and Russell, 2001). Transformants were selected from a LB agar plate containing erythromycin. Plasmid was extracted from the transformed *E. coli* and subjected to the sequencing analysis using the plasmid forward primer, pnzf (5'-TAGGAGGTAGTCCAAATGGC-3') and



Figure 1. Expression plasmid harboring the E. coli appA phytase gene.

the plasmid reverse primer, pnzr (5'-TGATTTACTGTATTCAGGAGGAG-3'). After the blast analysis and confirmation of the cloned fragment identity, plasmid was electroporated to L. lactis and L.salivarus as described earlier (Mason et al., 2005). The electroporated L. salivarus and L. lactis were cultured in MRS and M17 broth, respectively, for 3 hours. Then, they were spread on a solid medium containing erythromycin and incubated at the ambient temperatures until the transformants appeared. This process usually took 24 to 48 hours.

Detection of Expression by Reverse Transcription PCR (RT-PCR)

Transformants were confirmed by the direct colony PCR using primers pnzf/pnzr and RT-PCR to detect specific *appA* RNA. The total RNA was extracted from *appA2* transformants using Pars Toos (Mashhad, Iran) RNA extraction kit. cDNA was synthesized (RevertAid[™] First Strand cDNA Synthesis Kit, Fermentas) with DNase-treated RNA and subjected to PCR using E1/K1 primers. The PCR product was analyzed by electrophoresis on 1% agarose gel.

Determination of Plasmid Stability

The stability of plasmid in recombinant cells was investigated by the Bates method(1989). Briefly, the transformed bacteria were grown in MRS or M17 broth medium without any antibiotic and maintained at the mid-log phase throughout 30 generations by refreshing bacteria. At the appropriate generation, bacteria were diluted serially and plated onto the medium with or without erythromycin to determine the rate of plasmid loss.

Purification of Recombinant Protein

Transformed bacteria were grown to the middle exponential phase (OD_{600} = 0.4 for *L*.

lactis) and induced with lactose (2%) for 3 h prior to the harvest. Cells were washed with Tris-HCl 50 mM (pH= 7) and disrupted by sonication for 10 minutes (3 seconds pulse and 1 sesond rest). Then, all operations were carried out at 4°C. The cell debris was removed by centrifugation and the cell extract was mixed with saturated ammonium sulfate to obtain a final concentration of 25%. The mixture was incubated under agitation for 45 minutes and then centrifuged at 9,000× g for 25 minutes. The supernatant was mixed with a volume of saturated ammonium sulfate to reach 75% saturation and incubated under agitation for 12 hours. The mixture was centrifuged at 9,000×g for 25 minutes. The pellet was then dissolved in 5 mL 25 mM Tris-HCl with pH 7 and dialyzed overnight against the same buffer. The dialyzed sample was loaded onto a DEAE-Sepharose column equilibrated with 25 mM Tris-HCl, pH 7. After washing the column with 25 mL of the same buffer, the bound proteins were eluted with 25 mM Tris-HCl, pH 7 containing 1M NaCl. The fractions with a 46 kDa band corresponding to the phytase on the polyacrylamide gel were pooled and dialyzed against 25 mM Tris-HCl, pH 7.5 overnight for further analysis.

Phytate Zymography Analysis of Recombinant Bacteria

SDS-PAGE (10%) was performed as described by Laemmli (1970). The sample buffer did not contain any 2-mercaptoethanol to carry out non-denaturing electrophoresis. Protein samples were loaded in duplicate, symmetrically from both edges of the gel. One part of the gel was stained with Silver Nitrate (AgNO₃) and the other was subjected to zymography analysis (Bae *et al.*, 1999). A phytase plate assay was also carried out to evaluate phytase expression in the transformed bacteria. The recombinant bacteria were grown on a solid medium containing 1% sodium phytate at the proper time and temperature. After two days of growth, the

DOR: 20.1001.1.16807073.2016.18.4.9.3

colonies were washed off the agar surface and the Petri plates were flooded with a 2% aqueous cobalt chloride solution. After 5minutes incubation at the room temperature, the cobalt chloride solution was replaced with a freshly prepared solution containing equal volume of a 6.25% (w/v) aqueous ammonium solution and molvbdate 0.42% (w/v)ammonium vanadate solution. Following the 5-minutes incubation, ammonium molybdate-ammonium vanadate solution was removed and the plate was examined for cleared zones in which the colonies had been grown.

Phytase Activity

Phytase activity was measured in the cell extraction using a colorimetric method based on the determination of the released inorganic phosphorus, as described by Yin *et al.* (2007). One phytase unit was defined as the amount of enzyme that released 1 μ mol of inorganic phosphorous from 25 mg sodium phytate within 1 minute (Han *et al.*, 1997).

Determination of Acid Tolerance

The tolerance of recombinant and native *L. lactis* to acidity was evaluated at critical pHs of 2.5 and 3. The incubation lasted for 4 hours. The bacterial cells were prepared and diluted according to Ehrmann *et al.* (2002), and the results were expressed as the mean of log_{10} of Colony Forming Unit (CFU).

Determination of Bile Salt Tolerance

To determine bile salt tolerance, recombinant and native *L. lactis* were grown in the presence of different dilutions of taurocholic acid (Fluka, Sigma-Aldrich GmbH, Buchs; cat.86339) and ox bile (Fluka, Sigma-Aldrich GmbH, Buchs; cat.70168). A volume of bacterium was cultured in M17 broth containing 2% and 0.3% ox bile. To evaluate the growth in the presence of taurocholic acid, the bacterium were cultured in M17 broth containing 0.0, 7, 14, 21 mmol L⁻¹ of sodium taurocholate. The Optical Densities (OD) were monitored for 12 hours with 1 hour interval. Experiments were performed in four different series within 96 wells plate.

RESULTS

Nucleutid Sequnces of appA

The total lengh of the phytase insert (appA) was 1.25 kbp. A Blast search of cloned fragment showed 99% similarity to the reported *E. coli* phytase sequence in the GenBank (accession number AM946981.2).

Verification of Transformation and Expression of Plasmid into *L. salivarus* and *L. lactis*

The expression plasmid pBU*appA* was electroporated into *L. salivarus* and *L. lactis* with an efficiency similar to that of pBU003 $(1.48 \times 10^2 \text{ v } 2 \times 10^2 \text{ transformants per } \mu \text{g}$ of DNA). The presence of *appA* gene in *L. salivarus* and *L. lactis* was verified by direct-colony PCR. In both bacteria, plasmid primers (pNZ set) amplified a 1.5 kbp fragment containing *appA* sequence. The bacteria not carrying plasmid did not display any band and those of plasmids without insert showed a 0.25 kbp band.

Specific *appA* mRNA was also detected in transformed cells that showed positive results in direct-cloning PCR. *L. lactis* displayed a 1.3 kbp band by performing PCR, with synthesized cDNA serving as the template. In contrast, *L. salivarus* showed no specific RNA for *phytase* gene (Figure 2). Therefore, the plasmid instability in *L. salivarus* was suspected and analyzed.

Plasmid Stability

The stability of the recombinant plasmid in *L. salivarus* and *L. lactis* were assessed in the absence of antibiotic. In contrast to *L. salivarus*, more than 90% of *L. lactis*



Figure 2. Detection of specific *appA* RNA by polymerase chain reaction of cDNA. Lane1: 1 kb molecular marker; Lane 2: Negative control; Lane 3: Positive control; Lane 4: cDNA of *L. lactis,* and Lane 5: cDNA of *L. salivarus.*

colonies grown in the absence of antibiotic remained resistant to erythromycin even after 30 generations, as shown in Figure 3. Instability of plasmid in *L. salivarus* was confirmed by the direct-colony PCR so that no plasmid could be detected in bacterial cells after five generations.

Zymography Assay

The enzymatic activity of the recombinant *L. lactis* was confirmed by zymography assay. In zymographic analysis of acrylamid gel containing sodium phytate, the lysate of recombinant *L. lactis* revealed a 46 kDa band with phytase activity. A similar band was

detected on SDS-PAGE stained using silver nitrate (Figure 4). Also, phytase plate assay showed enzyme activity on the growth zone of *L. lactis* (Figure 5).This suggested that the *E. coli* phytase could be functionally expressed by *L. lactis* harboring pBU*appA*.

Phytase Activity Measurement

Phytase activity was measured in cell extracts of *L. lactis* (Table 2). A commercial *E. coli* phytase was also used as the standard. No activity was found in the culture supernatant fluid of *L. lactis* and *L. salivarus*. No phytase activity was detected in the cell extracts of *L. salivarus* as a result of plasmid instability. In



Figure 3. Plasmid stability in *L. salivarus* (■), and *L. lactis* (▲).



Figure 4. Silver nitrate stain (right) and zymogram experiment (left) on acrylamide gel. The samples loaded on the acrylamide gel symmetrically. Lane 1: Low molecular weight marker; Lane 2: Recombinant phytase, and Lane 3: Commercial phytase.



Figure 5. Petri dish containing M17 agar medium and 1% sodium phytate. Recombinant *L. lactis*had grown in the above part with a transparent zone. In the following part, non-recombinant bacteria were cultured and showed low transparency. The Petri dish was prepared by convert stained with using cobalt chloride and photographed by UV photography.

Table 2. Phytase activity of recombinant L.lactis and commercial enzyme.

	Recombinant phytase	Commercial phytase
Enzyme activity (U mL ⁻¹)	4.9± 0.16	220±1.2

the present study, phytase activity of nonrecombinant *L. lactis* was tested and any phytase activity was not observed (Figure 5).

Tolerance to Acidic pH

The colony count was not different between native and recombinant *L. lactis* at pH 3 during the experiment, but the recombinant *L. lactis* was more resistant to pH 2.5 in the first two hours, as compared to the native strain (Table 3).

Growth in Presence of Ox Bile and Taurocholate

The growth of the native and recombinant *L. lactis* was similar in the presence of two concentrations of ox bile (Figure 6). Both native and recombinant strains showed slow

probiotics to express specific enzymes can

have additional benefits for livestock

availability and reducing the cost of enzyme

commonly added to poultry feed to improve

phosphorus derived from plant sources

Phytase

increasing the nutrient

and availability

enzyme

is

of

production by

supplementation.

the

digestibility

	pH= 2.5					pH= 3		
Strain	1 h	2 h	3 h	4 h	2 h	3 h	4 h	
L. lactis (pBuappA)	4.23	3.50	2.22	1.10	7.60	6.06	6.92	
L. lactis	1.15	0.0	1.15	1.0	7.60	6.01	6.320	
Probablity	P < 0.05	P < 0.05	P > 0.05	<i>P</i> > 0.05	<i>P</i> >0.05	P > 0.05	<i>P</i> > 0.05	

Table 3. Effect of low pH on viability of recombinant and native *L. lactis* (log CFU mL⁻¹).

medium growth in the presence of concentrations of ox bile. Taurocholate did not significantly affect the growth of bacteria in exponential or stationary phases (Figure 7).

DISCUSSION



Figure 6. Growth of native and recombinant *L. lactis* in the presence of different ox bile extracts. (**A**) L. lactis, and (\blacksquare) L. lactis pBUappA.



Figure 7. Growth of native and recombinant *L. lactis* in the presence of taurocholate (21 mmol). (*L. lactis*; (\blacksquare) *L. lactis* pBU*appA*, and (\blacklozenge) In the absence of taurocholate.

The genetically engineered use of

In this study, the coding sequence of E. coli phytase was cloned and expressed in L. lactis with а molecular mass of approximately 44.6 kDa using a forward primer located at the upstream of the signal peptide cleavage site (Dassa et al., 1990) and a reverse primer located at the end of the gene. The amino acid sequence corresponding to appA showed 22 amino acid residues at the N-terminal as the signal peptide to direct their nascent chains into the periplasmic space. The molecular mass of the recombinant phytase (46.5 kDa) was consistent with other reports that had applied appA gene for cloning purposes (Rodriguez et al., 1999).

Determining the enzyme activity is a suitable approach to track expression of a recombinant enzyme. In this regard, phytase zymography was applied to the recombinant L. lactis, showing an active band of 46 kDa on acrylamide gel (Figure 4), while commercial E. coli phytase displayed two bands at 45 kDa, probably due to peptidase signal peptide. effect on the The recombinant phytase is a non-glicosilate protein which can maintains the phytase activity even after adding mercaptoethanol (the data are not shown). Extracted phytase enzyme from recombinant L. lactis showed an acceptable level of activity comparable to the studies in which this enzyme was transformed into other micro-organisms (Dassa et al., 1990; Lee et al., 2005)

The resistances to acidity and bile and probiotic properties of L. lactis have already been proved in some studies (Kimoto et al., 1999; Vinderola and Reinheimer, 2003). The resistance of recombinant L. lactis to acid was similar to that of native counterpart even in pH ranges lower than 2.5. Perhaps, pBUappA plasmid provides bacterium with some additional ability for resistance to low pH conditions. The recombinant bacteria exhibited slightly more absorbance than the grown bacteria in the absence of taurocholate in the stationary phase. It has been shown that the recombinant L. lactis has preserved its probiotic property the same as the native strain.

The transformation of L. salivarus was not successful due to the plasmid instability. This is a major obstacle for the utilization of recombinant microorganism in the а industry. The stability of plasmid depends on several factors including the mode of plasmid replication, plasmid size, medium composition, host inheritable background, culture medium, and temperature. (Kiewiet et al., 1993; Perez-Arellano et al., 2001; Mills et al., 2006). Two types of plasmid instability, i.e. segregation and structural, were assumed. The segregation instability describes the plasmid loss caused by unequal partition of the plasmid between daughter cells (defective partitioning), and the structural instability defines a plasmid population carrying structural deletions or rearrangements. The rolling circle replication type plasmids (like pWV01 family) often show a high degree of both instabilities due to the formation of single strand DNA (ss-DNA), intermediates and the formation of linear high molecular weight plasmid multimers (Gruss and Ehrlich, 1988; Gruss and Ehrlich, 1989; Leonhardt and Alonso, 1991). In the present study, given the sudden and quick reduction in recombinant cells, it seems that structural instability induces miss-plasmid in L. salivarus. It has been shown that both divergent transcription and the export of plasmid-specified proteins induce structural instability in derivate plasmids of pWV01 (Cordes et al., 1996). In the same line of research, Liu et al. (2007) showed that the deletion of lacA signal sequence in the recombinant plasmid of pNZ3004 could reduce structural plasmid instability (the plasmid drive from pWV01 family).

Our findings are consistent with the literature according to which genetically modified *L. lactis* maintained its probiotic character and could be used as a vehicle for delivering a specific protein to the gut environment (Steidler and Neirynck, 2005; Xin *et al.*, 2003). In addition, recombinant *lactobacilli* preserved their probiotic activities after acquiring enzyme production ability (Liu *et al.*, 2005; Liu *et al.*, 2007).

In the present study, *phytase* gene originating from *E. coli* was cloned and expressed in *L. lactis.* Phytase activity, determined in cell extracts of *L. lactis,* was not found in culture supernatant. Further *in vivo* evaluations of phytase degrading activity is suggested to confirm poultry applications. Also, the application of highly resistant plasmid with theta replication mechanism is suggested to achieve a successful transformation in *L. salivarus.*

ACKNOWLEDGEMENTS

The authors gratefully acknowledge the financial support of Research Administration of Mashhad University of Medical Sciences and the Excellence Centre for Animal Sciences, and Faculty of Agriculture, Ferdowsi University of Mashhad.

REFERENCES

- Augspurger, N. R., Webel, D. M. and Baker, D. H. 2007. An Escherichia Coli Phytase Expressed in Yeast Effectively Replaces Inorganic Phosphorus for Finishing Pigs and Laying Hens. J. Anim. Sci., 85: 1192-1198.
- Bae, H. D., Yanke, L. J., Cheng, K. -J. and Selinger, L. B. 1999. A Novel Staining Method for Detecting Phytase Activity. J *Microbiol. Meth.*, 39: 17-22.
- Bates, E. E., Gilbert, H. J., Hazlewood, G. P., Huckle, J., Laurie, J. I. and Mann, S. P. 1989. Expression of a *Clostridium Thermocellum Endoglucanase* Gene in *Lactobacillus plantarum. Appl. Environ. Microbiol.*, 55: 2095-2097.
- Chachaty, E. and Saulnier, P. 2000. Isolating Chromosomal DNA from Bacteria. In: "*The Nucleic Acid Protocols Handbook*", (Ed.): Rapley, R.. Humana Press, Totowa, New Jersey.
- Cordes, C., Meima, R., Twiest, B., Kazemier, B., Venema, G., van Dijl, J. and Bron, S. 1996. The Expression of a Plasmidspecified Exported Protein Causes Structural Plasmid Instability in *Bacillus subtilis*. J. *Bacteriol.*, **178**: 5235-5242.

- Dassa, J., Marck, C. and Boquet, P. 1990. The Complete Nucleotide Sequence of the *Escherichia coli* Gene *appA* Reveals Significant Homology between pH 2.5 Acid Phosphatase and Glucose-1-phosphatase. J. *Bacteriol.*, **172:** 5497-5500.
- Ehrmann, M.A., Kurzak, P., Bauer, J. and Vogel, R. F. 2002. Characterization of *Lactobacilli* towards Their Use as Probiotic Adjuncts in Poultry. *J. Appl. Microbiol.*, **92**: 966-75.
- Gruss, A. and Ehrlich, S. D. 1988. Insertion of Foreign DNA into Plasmids from Grampositive Bacteria Induces Formation of High-molecular-weight Plasmid Multimers. *J. Bacteriol.*, **170**: 1183-1190.
- 9. Gruss, A. and Ehrlich, S. D. 1989. The Family of Highly Interrelated Singlestranded Deoxyribonucleic Acid Plasmids. *Microbiol. Rev.*,**53:** 231-241.
- Han, Y., Wilson, D. B. and Lei, X. G. 1999. Expression of an Aspergillus niger Phytase Gene (phyA) in Saccharomyces cerevisiae. Appl. Environ. Microbiol., 65: 1915-1918.
- Han, Y. M., Yang, F., Zhou, A. G. and Miller, E. R. 1997. Supplemental Phytases of Microbial and Cereal Sources Improve Dietary Phytate Phosphrous Utilization by Pigs from Weaning through Finishing. J. Anim. Sci., 75: 1071-1025.
- Igbasan, F.A., Manner, K., Miksch, G., Borriss, R., Farouk, A., Simon, O. 2000. Comparative Studies on the *In vitro* Properties of Phytases from Various Microbial Origins. *Arch. Anim. Nutr.*, 53: 353-373.
- 13. Kerovuo, J. and Tynkkynen, S. 2000. Expression of *Bacillus subtilis* Phytase in *Lactobacillus plantarum* 755. *Lett. Appl. Microbiol.*, **30**: 325-329.
- Kiewiet, R., Kok, J., Seegers, J. F., Venema, G. and Bron, S. 1993. The Mode of Replication Is a Major Factor in Segregational Plasmid Instability in *Lactococcus lactis. Appl. Environ. Microbiol.*, **59:** 358-64.
- 15. Kimoto, H., Kurisaki, J., Tsuji, N. M., Ohmomo, S. and Okamoto, T. 1999. Lactococci as Probiotic Strains: Adhesion to Human Enterocyte-like Caco-2 Cells and Tolerance to Low pH and Bile. *Lett. Appl. Microbiol.*, **29:** 313-6.
- 16. Laemmli, U. K. 1970. Cleavage of Structural Proteins during the Assembly of

the Head Bacteriophage T4. *Nature*, **227**: 2079-2085.

- Lee, S., Kim, T., Stahl, C. H. and Lei, X. G. 2005. Expression of *Escherichia coli AppA2* Phytase in Four Yeast Systems. *Biotechnol. Lett.*, 27: 327-334.
- Lei, X. G. and Stahl, C. H. 2001. Biotechnological Development of Effective Phytases for Mineral Nutrition and Environmental Protection. *Appl. Microbiol. Biotechnol.*, 57: 474-481.
- 19. Leonhardt H., Alonso J. C. 1991. Parameters Affecting Plasmid Stability in *Bacillus subtilis. Gene.*, **103**: 107-111.
- Liu, J.-R., Yu, B., Liu, F. -H., Cheng, K. -J. and Zhao, X. 2005. Expression of Rumen Microbial Fibrolytic Enzyme Genes in Probiotic Lactobacillus reuteri. Appl. Environ. Microbiol., 71: 6769-6775.
- Liu, J. -R., Yu, B., Zhao, X. and Cheng, K. -J. 2007. Coexpression of Rumen Microbial β-Glucanase and Xylanase Genes in Lactobacillus reuteri. Appl. Microbiol. Biotechnol., 77: 117-124.
- 22. Majidzadeh Heravi, R., Kermanshahi, H., Sankian, M., Nassiri, M. R., Moussavi, A. H., Nasiraii, L. R. and Varasteh, A. R. 2011. Screening of *Lactobacilli* Bacteria Isolated from Gastrointestinal Tract of Broiler Chickens for Their Use as Probiotic. *African* J. Microbiol. Res., 5: 1858-1868.
- Mason, C. K., Collins, M. A. and Thompson, K. 2005. Modified Electroporation Protocol for *Lactobacilli* Isolated from the Chicken Crop Facilitates Transformation and the Use of a Genetic Tool. *J. Microbiol. Method.*, **60**: 353-63.
- 24. Mierau, I. and Kleerebezem, M. 2005. Ten Years of the *Nisin-Controlled* Gene Expression System (NICE) in *Lactococcus lactis*. *Appl. Microbiol. Biotechnol.*, **68**: 705-717.
- 25. Mills, S., Mcauliffe, O. E., Coffey, A., Fitzgerald, G. F. and Ross, R. P. 2006. Plasmids of Lactococci: Genetic Accessories or Genetic Necessities? *FEMS Microbiol. Rev.*, **30:** 243-273.
- Perez-Arellano, I., Zuniga, M. and Perez-Martinez, G. 2001. Construction of Compatible Wide-host-range Shuttle Vectors for Lactic Acid Bacteria and *Escherichia coli. Plasmid*, 46: 106-116.
- Pillai, P. B., O'connor-Dennie, T., Owens, C. M. and Emmert, J. L. 2006. Efficacy of an *Escherichia coli* Phytase in Broilers Fed

Adequate or Reduced Phosphorus Diets and Its Effect on Carcass Characteristics. *Poult. Sci.*, **85:** 1737–1745.

- Rodriguez, E., Han, Y. and Lei, X. G. 1999. Cloning, Sequencing, and Expression of an *Escherichia coli Acid Phosphatase/Phytase* Gene (appA2) Isolated from Pig Colon. *Biochem. Biophys. Res. Commun.*, 257: 117-123.
- 29. Sambrook, J. and Russell, D. W. 2001. *Molecular Cloning : A Laboratory Manual.* Cold Spring Harbor Laboratory Press, New York.
- 30. Stahl, C. H., Roneker, K. R., Pond, W. G. and Lei, X. G. 2004. Effects of Combining three Fungal Phytases with a Bacterial Phytase on Plasma Phosphorus Status of Weanling Pigs Fed a Corn-soy Diet. *Poult. Sci.*, 82: 1725–1731.
- Steidler, L. and Neirynck, S. 2005. Genetically Modified Probiotics. In: "Probiotics and Prebiotics: Scientific Aspects", (Ed.): Tannock, G. W.. Horizon Scientific Press. Cister Academic press, London.
- Van Rooijen, J. R., Gasson, M. J. and Vos, W. M. D. 1992. Characterization of the *Lactococcus lactis* Lactose Operon Promoter: Contribution of Flanking Sequences and LacR Repressor to Promoter Activity. J. Bacteriol., **174:** 2273-2280.
- 33. Veum, T. L., Bollinger, D. W., Buff, C. E. and Bedford, M. R. 2006. A Genetically Engineered *Escherichia coli* Phytase Improves Nutrient Utilization, Growth Performance, and Bone Strength of Young Swine Fed Diets Deficient in Available Phosphorus. J. Anim. Sci., 84: 1147-1158.
- 34. Vinderola, C. G. and Reinheimer, J. A. 2003. Lactic Acid Starter and Probiotic Bacteria: A Comparative "In vitro" Study of Probiotic Characteristics and Biological Barrier Resistance. Food Res. Inter., 36: 895-904.
- 35. Xin, K.-Q., Hoshino, Y., Toda, Y., Igimi, S., Kojima, Y., Jounai, N., Ohba, K., Kushiro, A., Kiwaki, M., Hamajima, K., Klinman, D. and Okuda, K. 2003. Immunogenicity and Protective Efficacy of Orally Administered Recombinant *Lactococcus lactis* Expressing Surface-bound HIV Env. *Blood*, **102**: 223-228.
- Xiong, A. S., Yao, Q. H., Peng, R. H., Han, P. L., Cheng, Z. M. and Li, Y. 2005. High Level Expression of a *Recombinant Acid*

[DOR: 20.1001.1.16807073.2016.18.4.9.3

Phytase Gene in Pichia pastoris. J. Appl. Microbiol., **98:** 418-428.

- Yin, Q. Q., Zheng, Q. H. and Kang, X. T. 2007. Biochemical Characteristics of Phytases from Fungi and the Transformed Microorganism. *Anim. Feed Dci. Tech*, **132**: 341-350.
- Zuo, R., Chang, J., Yin, Q., Chen, L., Chen, Q., Yang, X., Zheng, Q., Ren, G. and Feng, H. 2010. *Phytase* Gene Expression in *Lactobacillus* and Analysis of Its Biochemical Characteristics. *Microbiol. Res.*, 165: 329-335.

ساخت یک باکتری پروبیوتیک لاکتیک اسید بیان کننده آنزیم فیتاز مقاوم به اسید

ر. مجیدزاده هروی، م. سنکیان، ح. کرمانشاهی، م. ر. نصیری، ع. هروی موسوی، ل. روزبه نصیرایی، و ع. ر. وارسته

چکیدہ

استفاده از پروبیوتیکهای مهندسی ژنتیک شده به دلیل اینکه دسترسی مواد مغذی را می افزاید و هزینه های مربوط به مصرف مکملهای آنزیمی در خوراک را کاهش میدهند توجه ویژهای را در صنعت طیور به خود جلب کرده است. بطور معمول آنزیم فیتاز به منظور افزایش قابلیت هضم و دسترسی فسفر گیاهی به خوراک طیور اضافه می گردد. برای ساخت پروبیوتیکی با قابلیت تجزیه فیتات، ژن فیتاز

(appA)از ایکولای به دو باکتری پروبیوتیک لاکتوباسیلوس سالیواروس و لاکتوکوکوس لاکتیس انتقال داده شد. به دلیل ناپایداری پلاسمیدی درلاکتوباسیلوس سالیواروس بیان ژن در باکتری مزبور صورت نگرفت. بیان ژن appA در لاکتوکوکوس لاکتیس بوسیله روشهای تعیین RNA اختصاصی و زیموگرافی مورد بررسی قرار گرفت. آنزیم فیتاز از عصاره سلولی لاکتوکوکوس لاکتیس جدا شد و یک باند ۴۶ کیلودالتون را روی ژل آکرلامید با استفاده از SDS-PAGE نشان داد. زیموگرام فعالیت آنزیمی باند مزبور را تایید کرد. فعالیت آنزیمی U/ml 4.9 عصاره سلول لاکتوکوکوس لاکتیس بود. تعیین گردید. رشد باکتری بومی و نوترکیب در حضور غلظتهای متفاوت عصاره صفراوی یکسان بود.