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

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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 hexakis-dihydrogen 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

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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 evolutionary pressures (Kerovuo 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 (appA) from *Aspergillus ficuum* 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. *Cremoris* MG1363 (NZ9000)	pepN::nisRnisK	(Mierau and Kleerebezem, 2005)
Lactococcus lactis pBu003	pepN::nisRnisK; carrying pBu003	This study
Lactococcus lactis pBu*appA*	pepN::nisRnisK; carrying pBu*appA*	This study
Lactobacillus salivarous	Isolated from gastrointestinal tract of broiler chickens	(Majidzadeh Heravi *et al.*, 2011)
E. coli MC1061	araD139, Δ(ara, leu)7697, ΔlacX74, galU-, galK-, hsr-, hsm+, strA	MoBiTec GmbH, Germany
Plasmids		
pNZ3004	Cm^{r}Em^r; *E. coli-lactobacillus* shuttle vector; 4.9 kb	(van Rooijen *et al.*, 1992)
pBu003	Cm^{r}Em^r; *E. coli-lactobacillus* shuttle vector; 5.0 kb	This study
pBu*appA*	Cm^{r}Em^r; pBu003 containing *E. coli appA* gene, 6.3 kb	This study

medium. To achieve selective media, the media were supplemented with 5, 10 and 400 μg 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 *Sal*I-*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*CTCGAG*TCTGCATTCGCTCAG AGTGAG-3′) and the reverse primer K1 (5′-GGTTT*GCGGCCGC*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 *Xho*I-*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
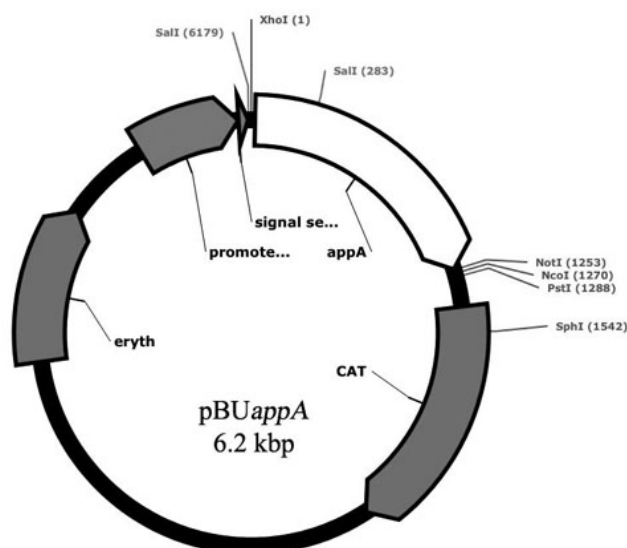
Figure 1. Expression plasmid harboring the *E. coli appA phytase* gene.

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 ($AgNO_3$) 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

colonies were washed off the agar surface and the Petri plates were flooded with a 2% aqueous cobalt chloride solution. After 5-minutes 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 molybdate solution and 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 1μmol 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 (1.48×10^2 v 2×10^2 transformants per μg 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*
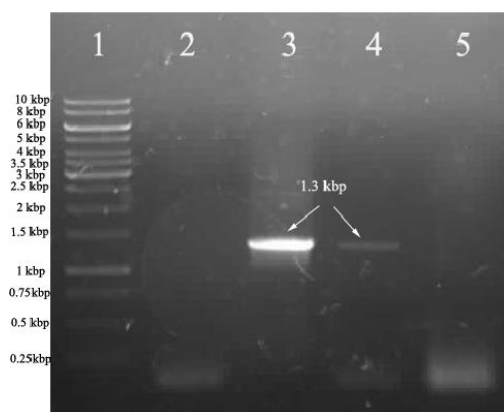
Figure 2. Detection of specific *appA* RNA by polymerase chain reaction of cDNA. Lane1: 1 kb molecular marker; Lane 2: Negative control; Lane 3: Positive control; Lane 4: cDNA of *L. lactis,* and Lane 5: cDNA of *L. salivarus*.

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.

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*.

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

## 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
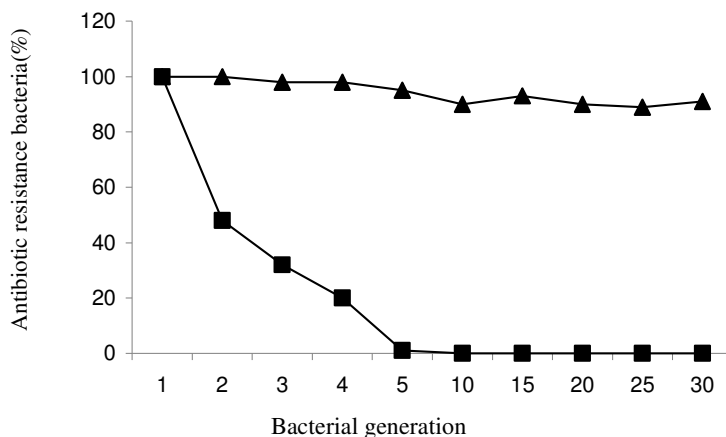
Figure 3. Plasmid stability in *L. salivarus* (■), and *L. lactis* (▲).
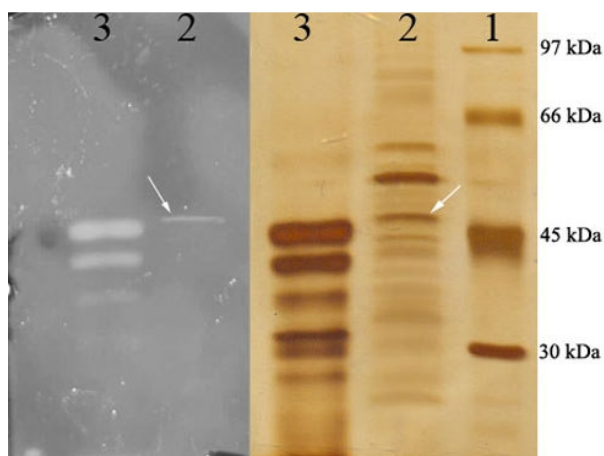
Figure 4. Silver nitrate stain (right) and zymogram experiment (left) on acrylamide gel. The samples loaded on the acrylamide gel symmetrically. Lane 1: Low molecular weight marker; Lane 2: Recombinant phytase, and Lane 3: Commercial phytase.
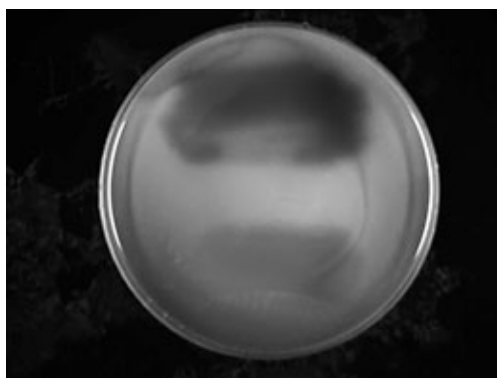
Figure 5. Petri dish containing M17 agar medium and 1% sodium phytate. Recombinant *L. lactis* had grown in the above part with a transparent zone. In the following part, non-recombinant bacteria were cultured and showed low transparency. The Petri dish was prepared by convert stained with using cobalt 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 non-recombinant *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

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

Strain	pH= 2.5				pH= 3		
	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	*P*> 0.05	*P*> 0.05	*P*> 0.05	*P*> 0.05

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

DISCUSSION

The use of genetically engineered probiotics to express specific enzymes can have additional benefits for livestock production by increasing the nutrient availability and reducing the cost of enzyme supplementation. Phytase enzyme is commonly added to poultry feed to improve the digestibility and availability of phosphorus derived from plant sources (Pillai *et al*., 2006; Stahl *et al*., 2004).
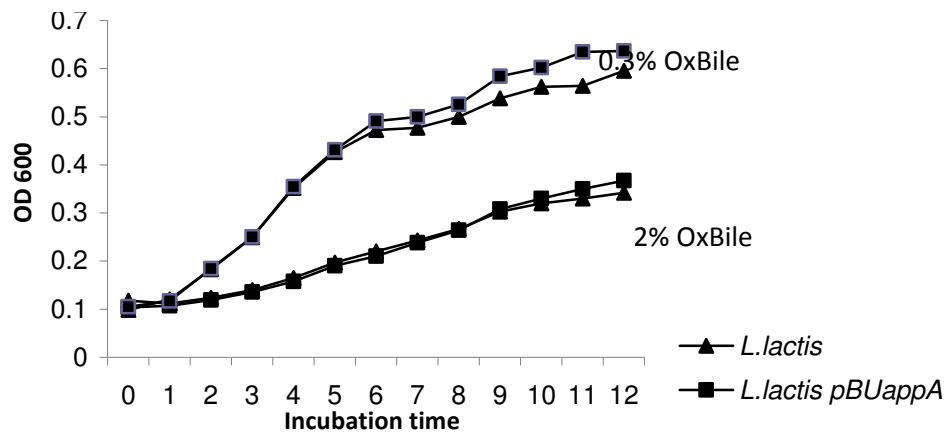
Figure 6. Growth of native and recombinant *L. lactis* in the presence of different ox bile extracts. (▲) *L. lactis*, and (■) *L. lactis* pBU*appA*.
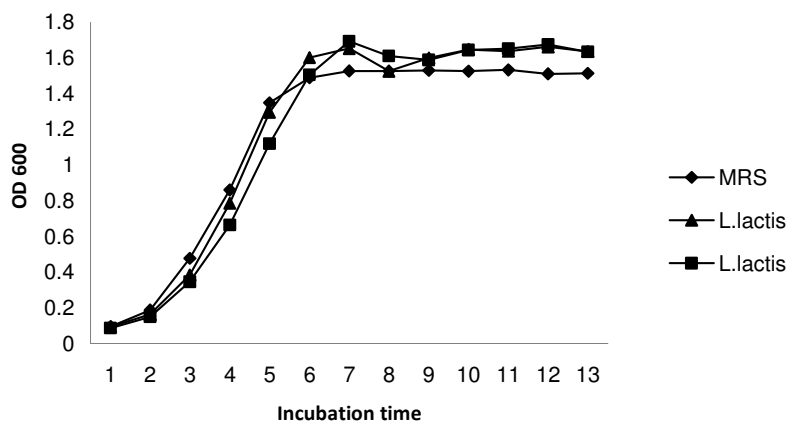
Figure 7. Growth of native and recombinant *L. lactis* in the presence of taurocholate (21 mmol). (▲) *L. lactis*; (■) *L. lactis* pBU*appA*, and (◆) In the absence of taurocholate.

In this study, the coding sequence of *E. coli* phytase was cloned and expressed in *L. lactis* with a 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 effect on the signal peptide. 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, pBU*appA* 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 a recombinant microorganism in the 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*.

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

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چکیده

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 می‌گردد. 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 لاکتوکوس 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 4.9 U/ml عصاره سلول لاکتوکوکوس لاکتیس تعیین گردید. رشد باکتری بومی و نوترکیب در حضور غلظت‌های متفاوت عصاره صفراوی یکسان بود.