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Expression of a Recombinant Therapeutic Protein, Lactoferrin, in PichiaPink™: a Powerful Antimicrobial Protein

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ABSTRACT: Lactoferrin (LF), an iron binding glycoprotein, is a part of the innate immune system that has a wide range of biological activities such as antimicrobial, antiviral, anticancer, antioxidant, anti-inflammatory activities, and several enzymatic activities as well. In this study, recombinant camel LF was expressed in the protease knockout yeast strain, PichiaPink™, using an inducible secretion cassette. After transformation, some of clones that had formed on the adenine free medium was selected for protein expression induction and bacterial growth inhibition test. A time-course study (24, 48, 96, 144 h) showed that inhibitory of the bacterial growth in all time point was highly significant, but the secreted protein concentration after 48 h alcohol induction was higher than other time points. Our results demonstrated that expressed recombinant camel LF using this system was secreted appropriately and it is active against *Staphylococcus aureus*.

Keywords: recombinant protein, Lactoferrin, yeast expression system, antibacterial

INTRODUCTION

The development of molecular biology and more accurate understanding the components of cellular and molecular mechanisms, particular recombinant DNA technology, led the experts used these findings in order to generate new combinations with large quantities and higher efficiency in microorganisms that did not exist in grams by use of foreign resources.

Application of recombinant DNA technology help to produce therapeutic proteins from diverse sources including more than 400 human therapeutic proteins and peptides that have therapeutic potential ability and yet more than 200 of them is approved by the US Food and Drug Administration (FDA). Insulin, albumin, interferon, human growth hormones and monoclonal antibodies have been considered as therapeutic (Demian and Vaishnav, 2009). Recently a high percentage of protein used in various industries are recombinant protein that thriving market is dedicated to them. Some of these enzymes includes protease, amylase, lipase, cellulase, lactase and xylanase, which are used in the food, detergents, textiles, leather, paper and polymers industries. Enzyme production efficiency, stability and activity increased by help of protein engineering (Demian and Vaishnav, 2009).

Diseases transmitted by food intake are a major problem in public health (Swaminathan *et al*., 2005). Control of microbial food pathogens is very important and several methods have been used to control or inhibit the growth of food borne pathogens including natural and synthetic antimicrobial agents (Payne *et al*., 1994). Nowadays consumer demand for natural methods that control foodborne pathogens is rising. Nisin and LF are example of compounds that can be used as a natural preservative in food products (Cleveland *et al*., 2001).

LF is an iron-binding glycoprotein that is part of the transferrin protein family. LF is produced by mucosal epithelial cells in various mammalian species, including humans, cows, goats, horses, dogs, and several rodents (Torres *et al*., 2006). This multifunctional protein also use as a natural preservative and has been found in mammalian secretions such as milk, colostrum, tears, saliva, nasal and bronchial fluid, hepatic bile, stomach, intestines, and urinary (Odeh *et al*., 2000; Oztas *et al*., 2005).

This protein is a part of the innate immune system (Legrang *et al*., 2010) and represents one of the first barrier against microbial agents invading that inhibit cell growth by sequester of iron and it has a wide range of biological activities such as antimicrobial, antiviral, anticancer, antioxidant, anti-inflammatory activities, immunomodulatory, regulation of gene expression, cell growth modulators and several enzyme activities (Wakabayashi *et al*., 1999; Payne *et al*., 1990; 1994). Researchers were looking for the easiest way to produce LF for several decades and currently the principal approach is purification of natural LF from milk and colostrum of several mammals, production of recombinant LF via bacterial and fungal expression systems and transgenic plants and animals.

Purification of LF from native sources such as milk is time-consuming and costly as well as the purification efficiency and protein purity is very low. Safety concerns about protein contamination with some dangerous animal pathogens are always raised. Hence recombinant proteins production in different systems such as mushrooms, yeasts, animals and transgenic plants has been also developed (Conesa *et al*., 2010).

Comparison of yeast with other expression systems, offers many advantages of recombinant protein production. Yeasts have relatively high growth rate, they has been described genetically and unlike bacteria are known to run many post-translational modifications. Working with yeast host to protein expression compared to the cells of insects and mammals is more convenient and less costly (Demian and Vaishnav, 2009). *Saccharomyces cerevisiae* and methylotrophic yeasts are the most important host among yeast expression systems. Methylotrophic yeast, *Pichia pastoris* has shown numerous potential to produce recombinant proteins. It has many advantages such as posttranslational modifications, easy to manipulate with high availability of intracellular and secreted recombinant protein and large-scale growth in simple and inexpensive medium (Demian and Vaishnav, 2009). However, after over 20 years, some limitations and disadvantages of the *P. pastoris* system have been gradually uncovered. The PichiaPink^{TM} strains are the mutants of *P. pastoris* which can be used for high-level and large-scale production of secreted bioactive recombinant proteins. The new strain, PichiaPink^{TM} offers the following advantages over traditional *P. pastoris* strains based protein expression system. (1) Selection of expression clones using ADE2 complementation (adenine auxotrophy). (2) The transformation efficiency of the PichiaPink^{TM} is in high rate and all transformants usually express the interest proteins. (3) Three protease knockout PichiaPink^{TM} strains help to reduce the impact of protease activity on degradation of the recombinant protein. (4) ADE2 complementation ensures higher stability of transformants during scale-up of protein expression. (5) The growth media is not complex and easy to screen transgenic PichiaPink^{TM} clones. (6) Intracellular protein expression using the pPINKk-HC (high copy) and pPINK-LC (low copy) vectors by removing the secretion signal sequences at the cloning step is possible as well as their general growth conditions and handling requirements are quite similar to *S. cerevisiae*. This system offers four ade2 strains. (1) Strain 1 is the ade2 knockout that is unable to grow on adenine free medium. (2) Strain 2 is pep4 knockout which prevents it from synthesize of proteinase A. (3) Strain 3 is prb1 knockout which is lack of proteinase B production ability and (4) Strain 4 is double knockout for both proteinases A and B, hence has the lowest protease activity among the PichiaPink^{TM} strains (Cregg *et al*., 2000; Daly & Hearn, 2005).

Studies on Arabian camel LF (*Camelus dramedarius*) have showed that this protein has anti-bacterial and anti-viral activities (khan *et al*., 2001; Rel-Redwan and tabll, 2007; liao *et al*., 2012; Sohrabi *et al*., 2014). Investigations on natural LF isolated from the camel milk and recombinant LF produced by insect cell culture have been done. According to the mentioned reasons above, this study was conducted by the aim of recombinant LF production in PichiaPink^{TM} (strain 4) in order to food industry applications.

MATHERIAL AND METHODS

This study was conducted at Institute of Biotechnology, College of Agriculture, Shiraz University, Islamic Republic of Iran.

A. Strains and vectors

Escherichia coli strain Top10 was used as a cloning host for amplification and storage of construction of the recombinant vector. PichiaPink^{TM} strain 4 (Invitrogen, Carlsbad, CA, USA) was used for protein expression. Secretion expression vector pPinkα-HC (Invitrogen) was used for genetic cloning and extracellular protein expression. *Staphylococcus aureus* PTCC 1112(ATCC 6538) was obtained from the Iranian Research Organization for Science and Technology (IROST) in order to the antimicrobial assay.

B. Enzymes and reagents

Oligonucleotide primers were synthesized by MWG Biotech (Marburg, Germany). *Stu*I, *Kpn*I, *Bam*HI, T4 DNA ligase, PrimeSTAR *Pfu* DNA polymerase (Takara), dNTPs, and DNA marker were purchased from Fermentas (Hanover, MD, USA). Plasmid and gel DNA extraction kits were purchased from Vivantis (Selangor Darul Ehsan, Malaysia). Anti-His tag antibody was obtained from BioLegend (San Diego, CA, USA).

C. Cloning of LF

Cloning of *LF*(cDNA encoding the *Camelus dromedaries* lactoferrin (*cLf*) was isolated from mammary gland by Sohrabi *et al.,* 2014) into the pPINK α-HC vector was done by using specific primers that were designed based on *LF* sequence (NCBI accession number; KF915308) using Allele ID 6 software. These primers were called LacPichF and LacPichR (LacPichR with *Kpn*1 site at the 5′ end). In addition to, to cloning verification partial primers were used (Table 1). PCR was performed to amplify gene of interest (Table 2). In order to increase cloning efficiency, PCR product was purified by Gel DNA Recovery Kit based on Vivantis procedure.

Table 1: Gene specific primers and their sequences.

Name	Primer sequence	product Size
LacPich F	CCTATGAAGCTCTTCTTCCCCGCCC	2150bps
LacPich R	CGGGGTACCTTAGTGGTGGTGGTGGTGGTGCCTCATCAGGAAGGCGC	2150bps
Lac-Part-F	GCTCCGCCAACAGGCTACTT	237bps

Table 2: The details of PCR reaction to amplify gene of interest.

Component	Amount
5x PrimstarBuffer+ $MgCl_2$	5uL
Primstar Pfu	0.25uL
DNTPs(10Mm)	2uL
LacPich F	1uL
LacPich R	1uL
Sterile water	To 25uL
Template	1uL

Table 3: Restriction digestion reaction of pPinkα-HC.

Component	Amount
pPinkα-HC(0.5ug/ul)	2uL
5x Restriction Enzyme Buffer	2uL
Sterile water	up to 20uL
StuI(1units/uL)	1uL
KpnI(1units/uL)	1uL
Incubate for 4 hours at 37^{o}c	

To insert desired gene in pPINKα-HC expression vector, digestion reaction was performed by *Kpn*I and *Stu*I restriction enzymes as the following condition (table 3). Then, the digested product was used in the ligation reaction. The ligation reaction with 20 µl final volume including the target gene and expression vector was done according to the manufacturer's protocol and was incubated at 22^{o}C. After 16 h, the ligation product was transformed to the competent *E. coli* cells by freeze and thaw method. After 1 hour, the transformed cells were cultured on LB medium supplemented with ampicillin 100 mg/L and were incubated over night at 37^{o}C. PCR was done with *LF* specific primers to verify the transformed colonies that were formed on the selection medium. Plasmid extraction was done from positive and was digested by *Bam*HI for further cloning verification.

D. PichiaPink^{TM} *transformation and PCR screening*

The purified recombinant plasmid was linearized by *Spe*I. Then, the transformation was continued according to the next proceduresFirst each strain (working glycerol stock) was spreaded on YPD (Yeast extract, Peptone, and Dextrose) medium and was incubated at 28°C for one day to getting the single colonies. Then, one colony was inoculated into the 10 mL YPD medium in a sterile 125 mL baffled flask and was placed in shaker-incubator at 28°C for 1 day, 200 rpm. This was the starter culture for the next step. Since adequate aeration is important for a fifth flask volume we filled. Then, 20 µl of the starter culture was inoculated into the 100 mL of YPD medium in a sterile 1 liter flask and was shaked in shaker-incubator under the same conditions until optical density reached to 1.5 at wavelength 600 nm (approximately 10 h). In the next step, cells were collected by centrifugation at 1500 ×g at 4 °C for 5 min and the pellet was resuspended using 8 mL of ice-cold sterile buffer. Centrifugation was repeated and then it was resuspended in 2 mL of 1 M ice-cold sorbitol. The previous step was repeated, but the pellet was resuspended in 1.5 mL of ice-cold 1 M sorbitol. After centrifugation, the pellet was resuspended in 5 mL ice-cold distilled water. Finally, centrifugation was repeated and the pellet was resuspended in 350 µl ice-cold distilled water. These competent cells were kept on ice for the transformation step. Then, 30 µg of the linearized recombinant plasmid was added to 200 µl of the competent cells, mixed by pipetting slowly, transferred into an ice-cold 0.2 cm electroporation cuvette and was incubated on ice to increase of the transformation efficiency. The suspension was pulsed using electroporator according to Bio-Rad's instructions. After electroporation, 1 mL of ice-cold YPD medium was added to the cuvette, mixed by pipetting immediately, and incubated at 28 °C without shaking for 12 h. Finally, 300 µl of the putative transformant cells was spreaded on the PAD (Pichia Adenine Dropout) selection medium.

The genomic DNA was isolated from untransformed and transformed PichiaPink™ according to Invitrogen procedure. PCR analysis was done using specific primers, forward primer based on the α-factor signal and reverse primer based on the CYC1 on the vector, to determine integration of LF into the genome of PichiaPink™ strain 4. The positive transformed cells were transferred in the induction medium to expression of LF. In addition to, total RNA was isolated from induced cells according to Invitrogen procedure and non-induced cells were used as negative control. According to Fermentas protocol, cDNA synthesis was done using 3 µg of total RNA, 15 pmol dNTPs, 20 U RNase inhibitor, 100 pmol 18 mer oligo-dT and 200 U M-Mulv reverse transcriptase in a 20 µl final volume. The synthesized cDNA was used as the template for PCR reaction with Lac-Part F and LacPich R primers in order to verify if LF was induced and transcribed.

E. Expression of LF in induction medium

Confirmed transformant cells were inoculated in 10 mL of BMGY Medium (Buffered Glycerol-complex Medium; 1 % yeast extract, 2 % pepton, 100 mM potassium phosphate pH 6.0, 1.34 % YNB, and 1 % glycerol) in 125 mL baffled flask and then were incubated overnight at 28^{o}C with shaking at 200 rpm in order to provide optimal growth conditions. The cells were transferred to falcon 50 mL conical tubes and were centrifuged at 1500 ×g for 5 min at room temperature.

To induce the LF expression, first the pellets were resuspended in 1 mL induction medium, BMMY (Buffered Methanol-complex Medium-yeast expression medium; 1 % yeast extract, 2 % pepton, 100 mM potassium phosphate (pH 6.0), 1.34 % YNB, and 0.5 % methanol), and were maintained at 28^{o}C in shaker-incubator for overnight. And then, this culture was transferred to 140 mL BMMY in a 500 mL filtered baffled flask. To induce AOX1 promoter 1.5 % filtered methanol was added and induction process was continued up to 6 days. The sampling was done at time points; 1, 2, 3, and 6 days after induction. In detail, 500 µl, 10 mL, and 30 mL of each sampling containing the secreted protein were collected in order to SDS-page. RNA extraction and protein extraction respectively. Then, all samples were precipitated at 1500 ×g for 10 min at 4^{o}C.

Cell pellets and the supernatants were transferred to falcon 50 mL conical tubes and 1.5 ml tubes separately, freezed quickly in liquid N2, stored in -80^{o}C for subsequent analysis. SDS PAGE was done by both supernatant and cell pellet for protein expression analysis. According to MIC (Minimum Inhibitory Concentration) test results, the cells pellet of 96h after induction was selected and purified using guanidine column (QIA gene kit) for ELISA analysis. Total soluble protein concentration was determined using the Bradford method (Bradford, 1976).

F. Antimicrobial assay

Minimum inhibitory concentration (MIC) method was conducted to determine the rate of bacteria growth inhibition by condensed protein supernatant.MIC is important in diagnostic laboratories to confirm resistance of microorganisms to an antimicrobial agent and also to determine the potency of new antimicrobial agents (Yarizade *et al*., 2012). Therefore, Gram-positive bacteria, *S. aureus* was grown overnight and was diluted with LB medium to give final concentrations of 5×10^{5} colony-forming unit (CFU) ml^{-1}. Briefly, 50 µl of recombinant protein extractions were added into 150 µl LB medium inoculated with 1 µl of bacteria in each 96-plate wells and were incubated at 37^{o}C. After 24 h, OD_{600} was measured. In this study, two controls were used; one containing the protein of non-transgenic yeast and another was normal bacteria culture without any inhibitory agent (positive control).

G. SDS-PAGE analysis of protein Expression

Total soluble protein concentration was determined using the Bradford method (Bradford, 1976). Then the samples at different time points were analysis using polyacrylamide gel analysis. Briefly, 40 µl of the induced supernatants were applied for SDS-PAGE. The LF protein size was 80 kDa, so 12 % polyacrylamide gel with 5% stacking gel was used.

H. ELISA

According to MIC results, the cell pellets of 96h after induction was selected to protein purification using guanidine column (QIA gene kit)

Then, ELISA was performed with KCL 0.2 g/L، Na_2HPO_4 1.44 g/L، KH_2PO_4 0.24 g/L، NaCl 8 g/L and Tween-20 0.1% (1 mL). In detail, first the wells of polystyrene 96well plates were coated with 100 mL of extracted protein of transformed yeast and 100 µl of PBS as a control solution and then were incubated at 37^{o}C for 60 min (the extracted proteins on time points 1, 2 and 6 days were used). After stated period of time, ELISA plate washed three times with PBST buffer and PBS buffer, then coated wells were blocked by 200 µl of 1% BSA and was placed at 37^{o}C for 60 min. Washing was repeated as first stage and in the next stage 100 µl anti-histidine was added to each well of the ELISA plate and were incubated at 37 °C for 60 min. Then, washing was repeated. Finally, 100 µl of TMB was added to each ELISA well. The plate were placed in dark for 4 min since a colorful reaction was observed and the solution color was changed to blue. The reaction was stopped by adding sulfuric acid 2.5 M. Finally, absorption was measured at a 630 nm and450 nm by ELISA reader.

I. Statistical analysis

All tests performed in triplicate and data were analyzed using the Minitab14 software.

RESULT AND DISCUSSION

A. Cloning of LF

In order to prevent errors in nucleotide sequence of camel *LF* ORF, the gene amplification was done using PrimeSTAR *Pfu* DNA polymerase. Then, pPink α-HC vector and PCR product were digested by restriction endonucleases; StuI and KpnI and then were used for ligation reaction. After cloning, Ppink α-HC vector containing LF ORF was named Lac-AC1 (Fig. 1).

Colony PCR was done using LacPich F and LacPich R primers (fig. 2). Also extracted plasmid was digested by *Bam*H1. As a result, fragments of digested 1503 bp and 9401 bp support the accuracy of cloning. After sequencing of recombinant *LF*, it was compared with the data available in the NCBI database. Results showed that the homology was almost 100 %.

B. PichiaPink^{TM} *transformation and PCR screening*

Colonies formed on adenine free selection medium confirmed integration of the gene of interest in genome of PichiaPink^{TM}strain4 (Fig. 3). Some of colonies formed on the selection medium were selected for PCR screening. Extracted genomic DNA and synthesized cDNA were used as template (Fig. 4). The color of colonies shows the relative expression amounts of the protein of interest.

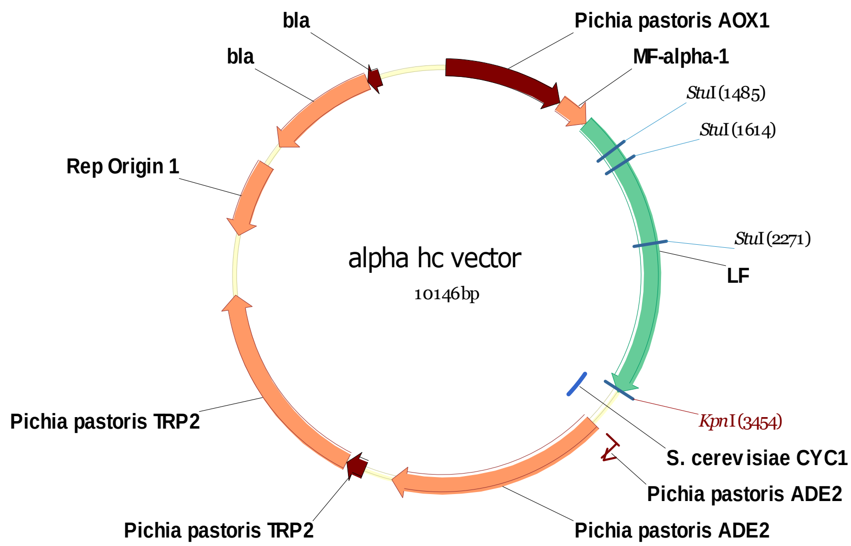

Fig. 1. Lac-AC1 Lactoferrin(LF) construct-PAOX1: 5'AOX1 promoter region -α-factor: α-mating factor secretion signal. Lactoferrin CDS. CYC1 TT: CYC1 transcription termination region. PADE2: ADE2 promoter region. ADE2: ADE2 ORF, TRP2: TRP2 gene, pUC ori: Oriental promoter of pUC, AmpR: Ampicillin (bla) resistance gene.

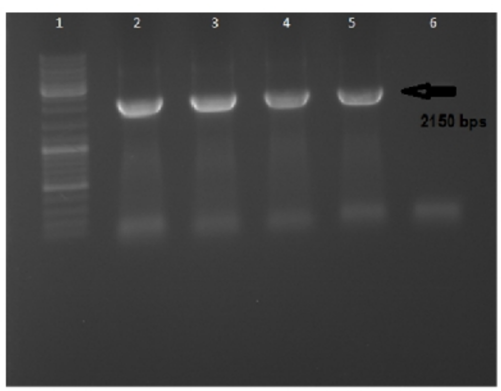

Fig. 2. PCR reaction using gene-specific primers to verify the cloning of LF (2150bp). (1)100bp ladder (2,3,4,5) selected transformant colonies (6) negative control.

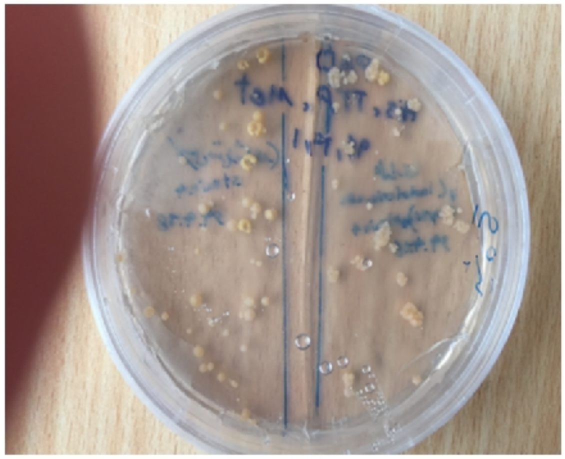

Fig. 3. *PichiaPink™* strain4 colonies formed on adenine free selection medium.

The pink colonies express very little ADE2 gene product, while the white ones express greater levels of the mentioned gene product, indicating that these white colonies contain more copies of integrated construct. Since colony color shows the expression levels of the gene of interest, implicitly, hence white colonies were selected for greater amounts of LF expression.

C. Antibacterial activity

Antimicrobial effect is one of the numerous biological activities of LF. Previous studies has suggested that iron chelation and the effect of limiting iron absorption are reasons for antimicrobial activity of LF (Zarember *et al.*, 2007). Furthermore, other reports have demonstrated the ability of LF in binding to other macromolecules (protein, DNA) (Baker and Baker, 2005) as well as direct interaction with bacterial and fungal membranes. Our results suggested that recombinant LF expressed in PichiaPink™ had appropriate antimicrobial activity, as it could inhibit bacteria growth about 70% in different time points in comparison with the control and nontransfromed yeast (Fig. 4).

LF is a member of the transferrin family (Baker and Baker, 2005) that its DNA sequence was isolated from different mammalian species such as human, mouse, cow, horse, camel, and etc. The members of this family sequester free iron, hence, it is the reason for their antimicrobial activities (Zarember *et al*., 2007).

The biological properties of LF are mediated by specific receptors on the surface of target cells. These receptors are typical for each cell type and can be found on surface of hepatocytes, macrophages and some bacteria like *S. aureus* (Adlerova *et al*., 2008).

Production of recombinant LF in other expression systems such as plant (Sohrabi *et al*., 2014) insect cells (Nakamura *et al*., 2001) and yeast (Chen *et al*., 2009; Wang *et al*., 2010; Chahardooli *et al*., 2014) with appropriate antimicrobial activities had been reported.. This study is the first successful report of recombinant LF production in PichiaPink™ expression system.

D. SDS-PAGE analysis

SDS-PAGE method is always used to determine and screen protein expression. The result of this study showed the successful production and secretion of recombinant LF, but a low concentration of the secreted protein was identified (Fig. 5).

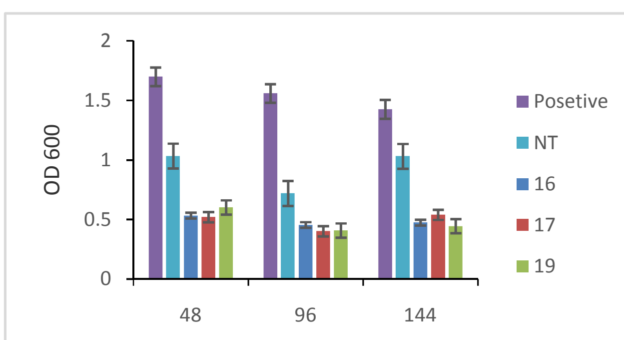

Fig. 4. MIC results show effective bacterial growth inhibitory of secreted LF from all three colonies.

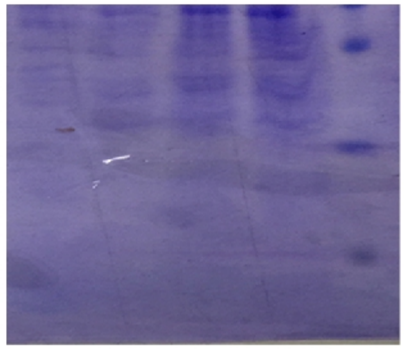

Fig. 5. SDS PAGE of 3 colony of lactoferrin secreting *P.Pink* strains. 1) negetive control, 2,3,4 transformed cells and 5) Protein ladder.

E. ELISA

These results confirmed the transformation and expression of LF in all colonies that were selected. (2) Also, existence of antimicrobial differences among time points and the highest levels of expressed protein was observed (Fig. 6). There was no significant difference between selected colonies with respect to the graph data (Fig. 7).

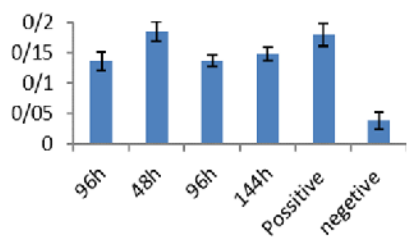

Fig. 6. ELISA results of different time points.

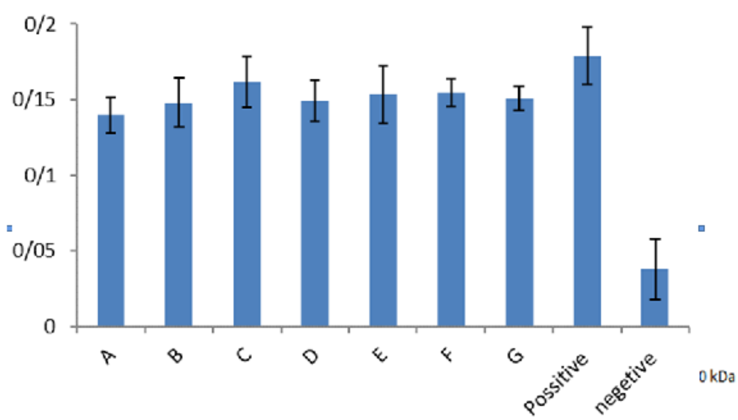

Fig. 7. ELISA results of different strains.

The PichiaPink™ expression system provides two different kits for secretion of recombinant protein. Since this yeast strains are protease knockout, higher amount of yield obtain because of low protein degradation in compare with other yeast expression systems. This new mentioned expression system provides an easier selection method as using adenine auxotrophy strains.

CONCLUSION

Since increasing of antibiotic-resistant bacteria strains and side effect of synthetic pharmaceuticals, evaluation of natural antimicrobial agents such as protein and peptides is valuable, nowadays. LF is one of these valuable peptides that has high antibacterial properties that was produced in various expression systems such as plant, insect cells, and yeasts.

PichiaPink™ expression system is better in compared with other expression systems because of mentioned advantages. Regarding to results of this study, PichiaPink™ expression system would be appropriated for LF production and application of this valuable protein in food industry.

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