EXPRESSION OF RECOMBINANT BOVINE LACTOFERRIN IN *PICHIA PASTORIS* AND ANTIMICROBIAL ACTIVITY OF ITS HYDROLYSATE

Thuy T.T. Trinh, Trang T. Nguyen, Thuy T. Nguyen, Phong Q. Truong*

Hanoi University of Science and Technology, Dai Co Viet Street, Hai Ba Trung District, Hanoi City, 122300, Vietnam

Abstract

The bovine lactoferrin (bLf) has been well-characterized as a multifunctional glycoprotein, which belongs to the transferrin family exhibiting multifunctional immunoregulation of antibacterial, antioxidant, anti-tumour and antiviral activities. In this study, the structure of pPICZαA::bLf$_{opt}$ was transformed into Pichia pastoris KM71H for heterologous production of lactoferrin under the control of the AOX1 promoter. The antimicrobial activity of the full rbLF and the pepsin-hydrolyzed rbLf against some pathogens were tested. The results showed that the optimized bLf gene encoding bovine lactoferrin was successfully transferred and expressed in Pichia pastoris KM71H. The expression of recombinant bovine lactoferrin was detected by SDS PAGE analysis and confirmed by Western blot. The recombinant bovine lactoferrin digested by Pepsin showed a higher percentage of inhibition than the whole molecular for three strains Candida albicans ATCC 10231, Staphylococcus aureus ATCC 6538P and Escherichia coli ATCC 11303 with the highest proportion of 50.71%; 56.76% and 21.11%, respectively. The successful expression of the rbLfopt genes in Pichia pastoris KM71H opens a prospect for developing research in natural antimicrobial agents.

Key words: *Bovine lactoferrin, expression, Pichia pastoris KM71H, antibacterial activity, hydrolyzed rbLf*

1. INTRODUCTION

Lactoferrin (Lf) has been considered as one of the major multifunctional glycoproteins and belongs to the transferrin family (Farnaud and Evans, 2003; Parc et al., 2017). Lf is well-characterized to be a single polypeptide chain folded into two lobes (the N-lobe and C-lobe), with 80 kDa and 703 amino acids in size and length, respectively (Garcia-Montoya et al., 2012). Lf is found in various exocrine secretions, such as milk, tears, nasal secretions, saliva, urine, uterine secretions and amniotic fluids, as well as in secondary granules of neutrophils (Weinberg, 2007). Lf has reportedly exhibited strong antibacterial, anti-fungal and antiparasitic activities with promising applications both in human and veterinary diseases (Faraji et al., 2017; Parkar et al., 2015). Particularly, Lf is capable of affecting the proliferation of several microorganisms with clinical importance, including Gram-positive and -negative bacteria strains, and viruses (such as human immunodeficiency virus), parasites, and fungi (Garcia-Montoya et al., 2012; Gonzalez-Chavez et al., 2009; Legrand et al., 2006).

Lf has its antimicrobial action not just in the form of the intact molecule but the mono-ferric lobes. Enzymatic digestion of Lf results in the generation of antimicrobial peptides which display antimicrobial properties, in some cases, with greater potency than the native Lf (Sinha et al., 2013). Until now, significant efforts have been made to isolate the antimicrobial peptides from Lf and demonstrate their antimicrobial and antivirus activities. Interestingly, approximately 60 queries related to Lf-derived peptides have been recently published in the MilkAMP database (Nielsen et al., 2017).

Regarding the potential functions of Lf, the demand for these proteins as an alternative source of antibiotics is increasing recently. Briefly, production of Lf from various sources can be obtained by mammal milk (Garcia-Montoya et al., 2012), like from humans, horses, pigs, goats, camel and sheep (Chahardooli et al., 2016; Han et al., 2007; Yen et al., 2009; Zhang et al., 2008). However, both of these methods are costly and have limitations in supplying sources. Recently, recombinant production presents an effective way because recombinant Lf can be produced quickly, efficiently, and can be scaled up. Among them, *Pichia pastoris*, with a strong regulator promoter, rapid growth rate, having post-

translation regulations, easy genetic manipulation and purification, is considered as one of the high potential systems of recombinant bioactive compounds (Ahmad et al., 2014; Chen et al., 2004). However, the usage of codon bias between the native gene sequence and the host has a significant impact on the expression level of recombinant protein. Codon optimization by using frequently used codons in the host is an efficient way to improve the expression level of the target gene (Wang et al., 2015).

In the previous study, the expression construction pPICZ αA*::bLf*$_{opt}$ was successfully produced in which *bLf*$_{opt}$ was optimized for expression in *P. pastoris* (Thuy et al., 2018*)*. This study aims at producing the recombinant *P. pastoris* carrying the *bLf*$_{opt}$; expressing this gene in *P. pastoris* KM71H. We next determined the antimicrobial activities of pepsin-digested rbLf against some pathogens.

2. MATERIALS AND METHODS

2.1. Chemicals, vectors and plasmids

Reagents for molecular biology methods were obtained from Sigma – Aldrich (USA), Invitrogen (USA) or Thermo Fisher Scientific (USA). The original nucleotide sequence of the *bLf* was obtained from the Genbank entry FJ589071.1 (2127 nucleotides from *B. taurus*). The optimized *bLf* gene sequence was synthesized by GenScript (USA). All primers used in this study were synthesized by IDT (USA) and listed in Table 1.

2.2. Strains and Media

The construction of *pPICZ αA::bLf*$_{opt}$ was produced and cloned into *E.coli* DH5α as previously described (Thuy et al., 2018). In order to express bLf in *P. pastoris*, strain KM71H was used and all media formulas were prepared as following to the EasySelect™ Pichia expression Kit Manual (Initrogen, USA). Transformants were cultured on the YPDS (1% yeast extract, 2% peptone, 2% dextrose, 1 M sorbitol, and 2% agar) agar plate. For selection of transformants, 100µg/mL of bleomycin was added to the media. In the screening experiments, BMMY (1 % yeast extract, 2 % peptone, 100 mM potassium phosphate (pH 6.0), 1.34 % YNB, and 0.5 % methanol) medium was used. Fermentation preparation was done according to the *Pichia* Fermentation Process Guidelines (Invitrogen).

2.3. Transformation of P. pastoris KM71H

The *Sac*I - linearized pPICZαA*::bLf*$_{opt}$*t* expression vector was added to 80 µl electro-competent *P. pastoris* KM71H competent cell suspension, mixed by pipetting slowly, transferred into an ice-cold 0.2 cm electroporation cuvette, and was incubated on ice to increase the transformation efficiency. The suspension was pulsed at 1.5KV and 3ms using a MicroPulser Electroporator (Bio-Rad, USA). Cells were immediately resuspended in 1 mL of ice-cold sorbitol and incubated at 28°C without shaking for one hour. Afterward, 300 µl cells were spread on the selective YPDS plates containing 100 µg/mL Bleomycin for selection and incubated at 28°C. Single colonies formed in the media after 3 - 5 days were chosen for screening by PCR with AOX1 and bLF primers (Table 1).

Table 1. The sequences of primers used in this study

Primer name	Sequences (5'-3')
bLF - F	GAATTCGCCCCAAGAAAGAACG
bLF - R	TCTAGACTAATGGTGGTGATGA
AOX1-F	GACTGGTTCCAATTGACAAGC
AOX1-R	GCAAATGGCATTCTGACATCC

bLF: Primers for detecting the bLf gene; F: Forward primer; R: Reverse primer; AOX1:Primers for detecting inserted gene at AOX1 promoter

2.4. Screening of P. pastoris KM71H transformants

Single colonies appeared on the selected media were obtained for culturing in the YPD (supplemented with 100µg/mL Bleomycin). DNA extraction was performed then applied for PCR with bLF and AOX1 primers. The thermo circle of PCR with bLF and AOX1 primers were set as following: (95^{o}C for 5 mins, 35 circles of 95^{o}C for 30 s, 59 ^{o}C for 45 s, 72 ^{o}C for 90 s then 72 ^{o}C for 5 mins) and (95^{o}C in 5 mins, 35 circles of 95^{o}C for 30 sec, 62 ^{o}C for 45 sec, 72 ^{o}C for 90 sec then 72 ^{o}C for 5 mins), respectively. PCR products were then applied to the 1% agarose gel for electrophoresis with 1kb DNA ladder (Intron, Korea).

2.5. Expression of bLf_{opt} gene in P. pastoris KM71H

Confirmed transformant cells were cultured in 2ml BMGY supplemented with bleomycin (100 µg/mL) and incubated at 28^{o}C, 250 rpm for 24h. The cells were harvested by centrifugation at 4^{o}C, 5000 rpm 5 mins then added to the 100ml BMMY medium to induce the expression of rbLf. Methanol 50% was then added to a final concentration of 0.5% (v/v) every 24 h for 4 days to remain the induction phase (Iglesias-Figueroa et al., 2016). The cells were then harvested by centrifugation at 5000 rpm in 10 mins, 4^{o}C for further analysis. Protein extraction was done for SDS PAGE, Dot blotting, Western blotting. Total concentration protein was determined by Bradford assay using bovine serum albumin (BSA) as previous standarded (Bradford, 1976).

2.6. Protein extraction by sonicator

After induction, the cells harvested by centrifuging were dissolved in PBS buffer then using 2 mm sonication probe with 60 % power for 600 seconds, 10'' on, 10'' off. Samples were put on ice. The samples were centrifuged to discard the broken cells, the supernatant was remained for further analysis.

2.7. Western blotting analysis

Proteins were separated by 12% SDS-PAGE gel, then were transferred to a 0.45 µm Nitrocellulose hybrid membrane (Chen et al., 2004). The membrane was blocked in TBS-Tween (25 mM Tris-HCl, pH 8.0, 140 mM NaCl, 2 mM KCl, and 0.05% Tween 20) containing 1% (w/v) non-fat dry milk shaking 1 hour. The membrane was then washed three times in PBS-Tween and was incubated for 2 hours at room temperature with diluted [1:5000 (v/v)] crude rabbit serum. The membrane was subsequently washed three times in PBS-Tween and was incubated with 1:30000 (v/v) peroxidase-conjugated secondary anti-rabbit antibody. The lactoferrin bands were visualized using a NBT/BCIP substrate.

2.8. Antibacterial activity of rbLf and rbLf hydrolysates digested by pepsin

The antimicrobial activity of rbLf and rbLfH was determined using a modification of the methods described by Bellamy et al. (1992). Briefly, purified rbLf was obtained as previously description (Thuat et al., 2020) then adjusted to the concentration of 2 mg/mL. Porcine pepsin (Serva, Germany) was added to rbLf solution at the various ratios. The mixture was incubated at 37^{o}C for 30 min with shaking 200rpm. Following the incubation, the reaction was terminated by incubation in 80^{o}C water bath for 15 min. After cooling, the solution was adjusted pH at 7.0 then sterilized by 0.22µm pore size filter.

To evaluate the antibacterial activity of rbLf and rbLf hydrolysates, the microbial culture of each strains (*Candida albicans* ATCC 10231, *Staphylococcus aureus* ATCC 6538P and *Escherichia coli* ATCC 11303) was incubated in falcon tubes containing suitable media with rbLf and rbLf hydrolysates at the final equivalent concentration of 1mg/mL. Bacteria and yeast in media without rbLf were used as control of growth.

3. RESULTS AND DISCUSSION

3.1. Transformation and expression of bLf_{opt} gene in P. pastoris KM71H

Transformation of pPICZαA::bLf_{opt} into P.pastoris KM71H

The plasmid pPICZαA*::bLf_{opt}* was restricted by *Sac*I then transferred into *P. pastoris* KM71H. The transforms were then inoculated on the selective agar plates supplemented with the antibiotic (100

µg/mL Bleomycin) for 3 - 4 days at 28°C. The non - transformed *P. pastoris* KM71H was used as control. After three days of cultivation, the plates appeared a total of 34 single colonies, the control plates did not appeared any colonies. Yeast cells *P. pastoris* are sensitive to the antibiotic Zeocin (Bleomycin) and will not grow on agar plates containing this antibiotic. According to the design of vector pPICZαA*::bLf*$_{opt}$ containing antibiotic resistance gene, thus *P. pastoris* carrying the structure of pPICZαA*::bLf*$_{opt}$ could be obtained on the antibiotic selective medium.

Next, seven out of 34 clones were randomly selected for genomic DNA extraction, and PCR was performed using bLF and AOX1 primers. As expected, all seven clones appeared a band of approximately 2 kb, of which be equivalent to the size of the *bLf*$_{opt}$ gene (Fig. 1 A). Due to the template used was genomic DNA, the obtained results demonstrated that seven positive clones carrying the target gene *bLf*$_{opt}$ were inserted into the *P. pastoris* genome (Jiang et al., 2008).

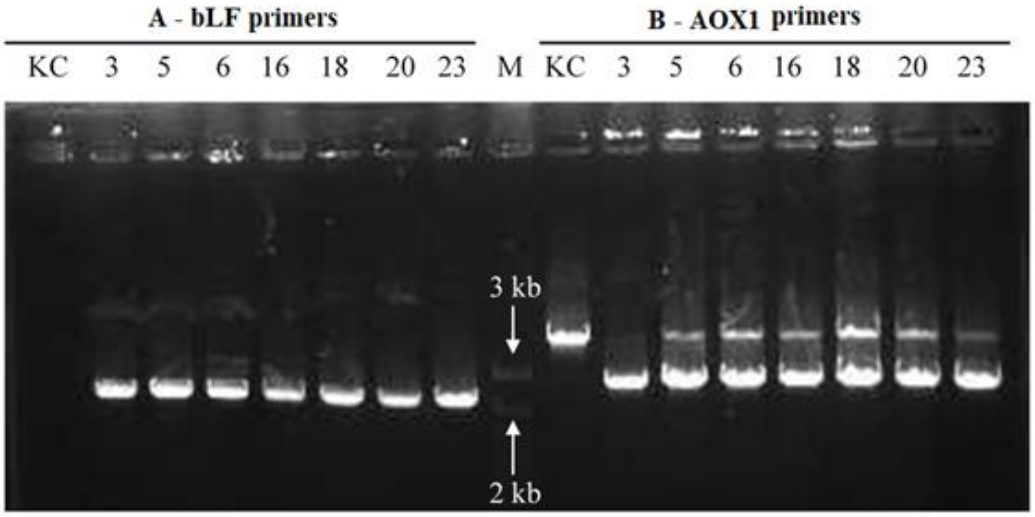


Fig. 1. Amplification of genome recombinant *P. pastoris* KM71H with bLF and AOX1primers

A: PCR results with bLf gene - specific primer; B: PCR results with AOX1 vector - specific primer
KC - negative control (*P. pastoris* KM71 H without bLf gene), M: marker

The amplicons of recombinant strains with AOX1 primers had two bands, about 2.6 kb and 3.6 kb, while the control strain showed only a band of approximately 3.6 kb (Fig.1B). This result was entirely consistent with the theoretical calculation and can be explained as follows: the vector pPICZαA*::bLf*$_{opt}$ was inserted into the 5'-AOX1 locus of the host genome. With this design, when using genomic DNA as a template for PCR, the AOX1 primers specifically pairs with two regions 5'-AOX1 and 3'-AOX1, so that the recombinant strains have two product bands with the corresponding size of 2.6 kb (2.1 kb of the target gene bLf$_{opt}$ is + 359 bp of the 5'-AOX1 terminus + 173 bp of the 3'-AOX1 terminus) and about 3.6 kb (nearly 1.6 kb of the AOX1 gene in the host cell and the 2.0 kb of inserted ARG4 gene). Non-recombinant strain have a band of about 3.6 kb. The Fig. 1 showed that all strains gave expected results. It is indicated that the construction of pPICZαA::*bLf*$_{opt}$ was successfully introduced into *P. pastoris* KM71H.

Expression of bLf$_{opt}$ *in P. pastoris KM71H*

In order to explore the expression of Lf in the *P. pastoris* KM71H, the recombinant clones were activated on the BMGY medium, then induced on the BMMY medium as detailed in the method description. Biomass was collected and analyzed for total protein. The SDS – PAGE analysis results showed that the protein band (about 75 kDa) appeared stronger in the recombinant lines than the control sample, non –

transformed strain (Fig. 2). Theoretically, the recombinant Lf is about 76.9 kDa in size, so the protein band that differs between the recombinant and control strain is assumed to be the recombinant.

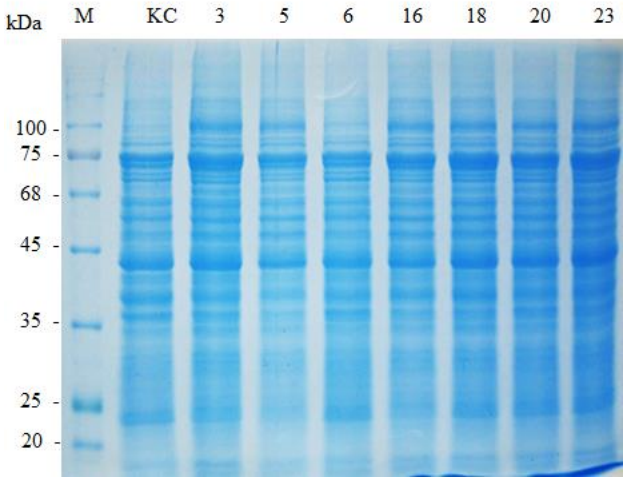


Fig. 2. SDS – PAGE of intracellular protein from seven recombinant *P. pastoris* KM71H clones and the control (non-transfromed *P.pastoris* KM71H).

M: Marker; KC: control sample; 3,5,6,16,18,20,23: seven transformed clones of *P.pastoris* KM71H

The successful expression of recombinant bLf was confirmed by Western blotting. Three clones showed strong bands (clone 3, 6, 16) in the SDS – PAGE gel were chosen for analysis with the control strain (non – transformed clone). Total protein extracted from three recombinant clones and the control were electrophoresed then transferred onto the nitrocellulose membrane and incubated with the two antibodies in which the primary antibody is rabbit anti-His-tag, secondary antibody is goat anti-rabbit IgG conjugated with alkaline phosphatase. The signal is visualized by the response with NBT/BCIP substrate. The results showed that the Western blot signal spectrum only appeared in three recombinant clones, while it was completely absent in the control samples (Fig. 3). This result is consistent with the theoretical calculation because, by design, recombinant lactoferrin has six amino acid residues Histidine attached to the C – terminus. In each lane of the recombinant clones, there were a major band of about 75 kDa and some smaller ones. The presence of some smaller fragments may be due to the proteolytic activity of lactoferrin leading to itself digestion (Massucci et al., 2004). Data suggested that lactoferrin was expressed in the recombinant clones. Iglesias-Figueroa also reported the recombinant bovine lactoferrin from *P. pastoris* was about 80 kDa (Iglesias-Figueroa et al., 2016).

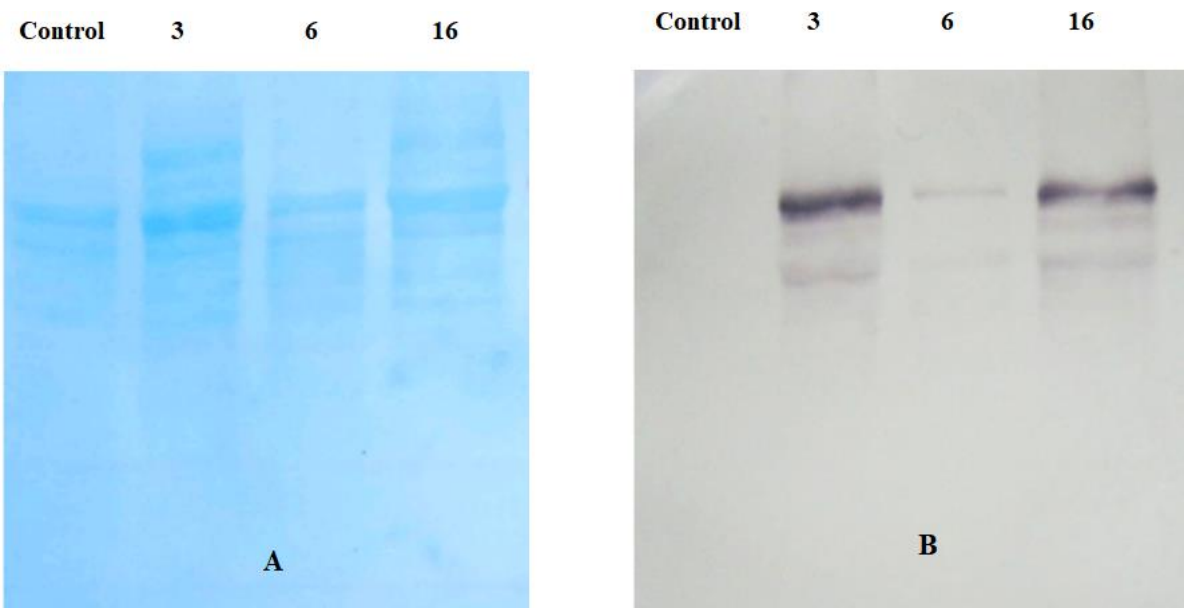


Fig. 3. Western blot analysis of recombinant bovine lactoferrin (rbLf) from three recombinant clones of *P. pastoris* KM71H (3, 6, 16) and the control (Non-transformed *P.pastoris* KM71H)

A - SDS – PAGE of total protein from 3 clones (3,6,16) and the control; B - Western blot signal of three clones (3,6,16) and the control

3.2. Effect of hydrolyzed recombinant lactoferrin from P. pastoris KM71H – 3 on microorganisms

Effect of the ratio of pepsin per rbLf for the digestion of rbLf

In our previous study, *P.pastoris* KM71H- 3 (clone 3) was selected to perform fermentation for obtaining rbLf. This protein was then purified and determined the biological activities. We evaluated the antibacterial activity of intact rbLf against five testing microorganisms in which rbLf inhibited only two strains of *M. luteus* ATCC 10240 and *B. subtilis* ATCC 6051 (Thuat et al., 2020). It has been hypothesized that the hydrolysate of rbLf could present a higher antimicrobial activity than the intact protein (Iglesias-Figueroa et al., 2016). In this study, we continued to investigate the antibacterial activity of the remaining three strains with hydrolyzed – rbLf.

Table 2. Table of ratio Enzyme/Substrate

No.	Amount of Lf (µg)	Ratio of Pepsin/Lf (U/mg)
1	500	5×10^{-4}
2	500	10×10^{-4}
3	500	50×10^{-4}
4	500	100×10^{-4}
5	500	200×10^{-4}

In order to obtain the rbLf hydrolysate, digestion of rbLf with pepsin was performed with the ratio of pepsin per rbLf varying from 5×10^{-4} U/mg to 200×10^{-4}U/mg (Table 2). The digestion solution obtained a broad range of peptides with the molecular mass focused mostly less than 35 kDa (Fig. 4).

Our study showed that the proportion of hydrolyzed rbLf increased gradually from lane 2 to 6 corresponding to the enzyme/substrate ratio in the reaction increasing from 5×10^{-4} U/mg to 200×10^{-4}

U/mg. The amount of Lf in lane 2 was almost not hydrolyzed, whereas Lf in lane 5 was incompletely hydrolyzed; lane 6 was almost completely hydrolyzed (Fig**.** 4). In this study, the pepsin - digested rbLf in lane 5 (rbLfH-1) and lane 6 (rbLfH-2) corresponding the ratio of pepsin per rbLf of 100×10^{-4}U/mg and 200×10^{-4}U/mg, respectively, were used to test the antibacterial potency against test microorganisms.

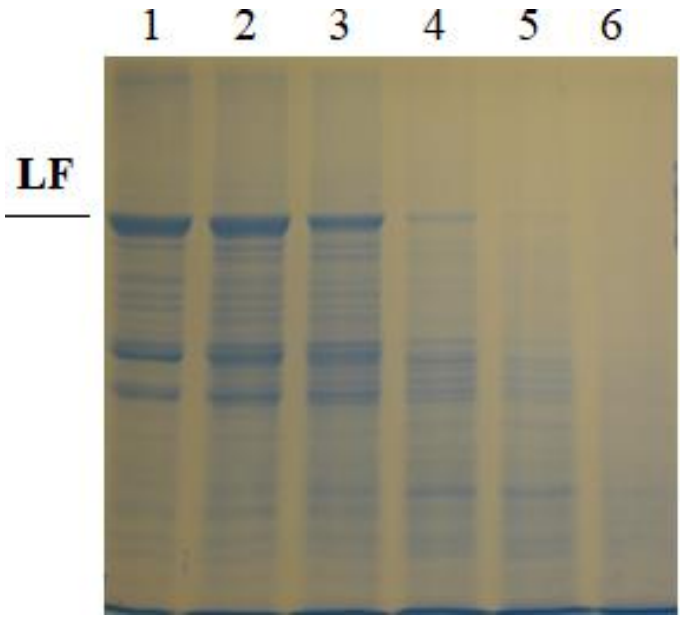


Fig. 4. Pepsin – digested rbLf

Lane 1 – purified rbLf; Lane 2 – 6: pepsin – digested rbLf with variable rate of pepsin/rbLf ; 2 - 5×10^{-4} U/mg; 3 - 10×10^{-4} U/mg; 4 - 50×10^{-4} U/mg; 5 - 100×10^{-4} U/mg; 6 - 200×10^{-4} U/mg)

Antimicrobial activities of recombinant lactoferrin digested by pepsin

The ability of hydrolyzed rbLf to inhibit the growth of *Candida albicans* ATCC 10231, S*taphylococcus aureus* ATCC 6538P and *Escherichia coli* ATCC 11303 were determined through the OD600 value. The percentage of inhibition against three tested strains of rbLf and their hydrolysates was calculated and presented in Fig. 5.

The results showed that the peptide hydrolysate of rbLf had a much greater antimicrobial activity than the whole molecular at a concentration of 1mg/mL for each pathogen tested. Recombinant bovine lactoferrin was just inhibitory 2.04% for *C. albicans* ATCC 10231 at the tested concentration after 16 hours of incubation, while hydrolyzed recombinant bovine lactoferrin had a higher percentage of inhibition of 42.77 and 50.71 with the rbLfH-1 and rbLfH-2, respectively (Fig. 5A).

For the *S. aureus* ATCC 6538P, the percentage of inhibition increased sharply from intact molecular of rbLf to the hydrolyzed rbLf at the concentration assayed. The rbLf hydrolysates were the most effective with *S. aureus* 6538P with the highest inhibition proportion of 56.76% for rbLfH-1 followed by rbLfH – 2 with a rate of 43.14%, while the rbLf showed the inhibitory of 0.89% (Fig. 5B).

Similar to *C. albicans* ATCC 10231 and *S. aureus* ATCC 6538P, *E. coli* ATCC 11303 was shown the percentage of inhibition for rbLf hydrolysate was higher than that of rbLf at the same tested concentration. There was a slight difference in antimicrobial activity for *E. coli* ATCC 11303 between rbLfH -1 and rbLfH-2 with the rate of 19.63 and 21.11, respectively (Fig. 5C). These figures for the inhibition rate of *E. coli* ATCC 11303 were the lowest among three strains.

Effect of pepsin – digested rbLf on the growth of pathogen strains in this study coincided with the observation of many researcher (Iglesias-Figueroa et al., 2016; Shin et al., 1998 ; Vorland et al., 1999). Previous studies report that pepsin-digested Lf has been shown to exert a more potent antimicrobial

effect than its parent molecule Lf (Lawyer et al., 1996, Odell et al., 1996). The increment can be explained that the proteolytic digestion of Lf by pepsin releases bioactive peptides; these smaller fragments can alter the permeability of bacterial membrane (Wakabayashi et al., 2006; Yamauchi et al., 1993).

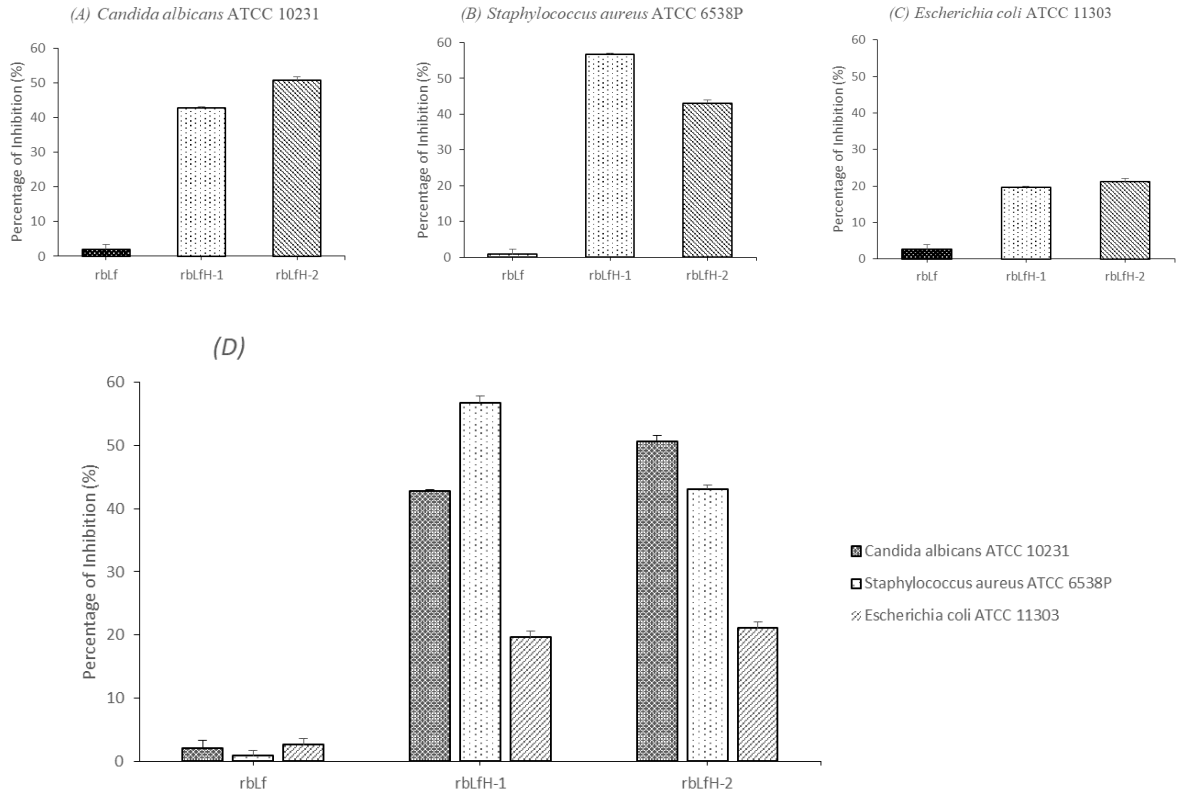


Fig. 5. Antibacterial activity of rbLf and rbLfH towards (A) Candida albicans ATCC 10231, (B) Staphylococcus aureus ATCC 6538P, (C) Escherichia coli ATCC 11303 and (D) Summary of all test strains.

4. CONCLUSION

This study indicated that the bLf_{opt} gene was successfully expressed in *Pichia pastoris* KM71H. The molecular weight of the recombinant lactoferrin in this host strain was around 75 kDa which was corresponding to theoretical calculation. Recombinant bovine lactoferrin hydrolyzed by pepsin exhibited much potential in antimicrobial activity against *Candida albicans* ATCC 10231, *Staphylococcus aureus* ATCC 6538P and *Escherichia coli* ATCC 11303. The further studies are required to upscale production of this protein for further application as antimicrobial agents to deal with the phenomenon of drug resistance in microorganisms.

Abbreviations

bLf_{opt} Optimal bovine lactoferrin gene

rbLf Recombinant bovine lactoferrin

rbLfH Recombinant lactoferrin hydrolysate

rbLfH -1 Pepsin hydrolysate of recombinant bovine lactoferrin at ratio 100×10^{-4}U/mg

rbLfH -2 Pepsin hydrolysate of recombinant bovine lactoferrin at ratio 200×10^{-4}U/mg

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