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Cloning and expression of plantaricin E and F genes of *Lactobacillus Plantarum* S34 isolated from Indonesia traditional-fermented meat *(Bekasam)*

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Abstract

Heterologous protein expression has been used in attemp to increase bacteriocins yields by less laborius process. In this study, the plnEF genes (530 bp) encoding plantaricin S34 have been identified and cloned to pGEMTeasy s vector. Plantaricin S34 gene had 99% similarity with all those plnEF locus of *Lactobacillus* references strain aligned. Furthermore, plnE peptide of plantaricin S34 had unique one amino acid substitution at position 33 (lysine > cysteine), while two amino acids were substituted at position 14 (alanine > serine) and 42 (valine > isoleucine) appeared on plnF peptide. PCR amplification of mature fragment of plnE and plnF gene produced the bands with length of approximatly 102 bp and 105 bp respectively. Moreover, Both of fused recombinant plnE and plnF peptide have been expressed heterologously in *E. coli* as protein fusion with thioredoxin-$(His)_{6tag}$ with given molecular mass of approximatly 21 kDa for each peptides. Overall, the partial operon of plnEFI loci of *L. plantarum* S34 has been characterizied and the plnEF genes that composed this operon was successfully cloned and produced as hetelogous recombinant peptides.

Introduction

Lactic acid bacteria are known to have ability to ferment many kind of food and feed based material. In natural process of fermentation, these saprophyte bacteria are responsible for preservation of fermented product. It was long believed that the anti-food spoilage and natural biopreservation excreted by LAB may be due to the production of a number of antimicrobial substances such as lactic acid, hydrogen peroxide, diacetyl and bacteriocins. *L. plantarum* species may produce a considerable number of bacteriocins and bacteriocin-like peptides, as illustrated by recent genetic and biochemical studies of *L. plantarum* C11. This strain produces at least two two-peptide and one one-peptide bacteriocin, as well as a bacteriocin-like secreted peptide pheromone that is necessary for activating the expression of genes involved in bacteriocin production (Diep *et al.*, 1995, 1996; Nes *et al*., 1996; Bruberg *et al*., 1997; Anderssen *et al.*, 1998). Furthermore, plantaricins, which its production rely on present of peptide pheromone, have naturally been produced by different strains of *L. plantarum*. These are such as plantaricins EF (PlnEF) and plantaricins JK (PlnJK) that belong to a large group of small, heat-stable, cationic and no lanthionine-containing peptides (Hauge *et al*., 1999; Moll *et al*., 1999).

Plantaricin peptides have potential application as promising compounds particullary in reducing the usage of antibiotics in agriculture and food industry to fight microbial infections and contaminations, thus problem related to antibiotic resistance could also be decreased gradually (Cleveland *et al.*, 2001). However, one of the major constraints have been the fact that low yield, and difficulty of purification process. Our earlier work on *L. plantarum* S34 has confirmed the low yield of purified peptides from the culture filtrate.

In attempt to increase the yield of bacteriocin peptides, their heterologous expression in procaryotic species have been widely studied (Richard *et al*., 2004; Klocke *et al*., 2005; Fimland *et al.*, 2008; Rogne *et al.*, 2009; Basanta *et al*., 2010; Fang *et*

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al., 2010; Borrero *et al*., 2012). By this powerful tool, not only the yield could be improved but also faster production, improved biological acitivity and less laborius process could be achieved. But until now, reports on the application of this technology especially at plantaricin expression and production still limited (Fimland *et al*., 2008; Rogne *et al*., 2009; Fang *et al.*, 2010). Many biological processes of the class II bacteriocins are well understood and their heterologous protein expression have been done using pET expression vectors. For example, Divercin V41 was overexpressed in pET32b using the T7 RNA polymerase promoter in the *E. coli* Origami (DE3) (pLysS) strain and was accumulated in the cell cytoplasm in a soluble anti-Listeria active form (Richard *et al*., 2004), while sakacin P was overexpressed in pET28a in *E. coli* BL21 (DE3) after induction with IPTG forming inclusion body but its activity against *Listeria monocytogenes* restored after resolubilization treatment (Chen *et al*., 2012). It seems that different in expression vectors used will impact on recombinant peptides properties especially their biological activity.

In this study, we cloned partial operon of plnEFI loci isolated from *L. plantarum* S34 (*Bekasam* origin) and characterized the correspondence genes, pln E and plnF, and expressed the genes individually using pET32a as the expression vector with major purpose is to express these peptides in *E. coli* to enhance the yield.

Materials and Methods

Bacterial strains and growth condition

E. coli strains used for cloning and protein expression, *E. coli* TOP 10 and *E. coli* BL21 (DE3) pLysS respectively, were grown in LB medium at 37°C, with agitation at 150 rpm. Agar plates were made by adding 1.5% (w/v) agar to the growth medium. For selection of *E. coli* transformants, chloramphenicol and amphicilin were added at concentration of 0.1 mg/ml respectively (Fimland et al., 2008).

Isolation of genomic DNA from bacteria

The total genomic DNA of L. plantarum S34 (this strain was kindly obtained from Dr. Apon Zaenal Mustopa, Research Center for Biotechnology, Indonesian Institute of Sciences, Indonesia) was isolated in small scale preparations from 5 ml of overnight culture grown at 37°C in MRS broth. The genomic DNA was extracted as previously described with modification (Liu *et al*., 2012). Bacterial cells were collected by centrifugation at 11,000 g for 10 min. The pellet was resuspended with 10 ml of TE buffer (10 mM Tris–HCl pH 8, 1 mM EDTA), 40 µl of lysozyme (60 mg/mL). The solution was then Incubated at 37ºC for 60 min and added with 200 µL 10% sodium dodecyl sulfate, 100 µL 5 M NaCl, 80 µL 10% CTAB was added. The solution then warmed at 68°C for 30 min and added an equal amount of chloroform then followed by centrifugation at 23,000 g for 10 min. The supernatant was harvested and an equal amount of ethanol was added. For 10 min centrifuged at 4 ºC, agitation at 23,000 g. The pellet was then harvested and air-dried, and then DNA was dissolved in TE buffer (10 mM Tris–HCl, 1 mM EDTA, pH 8.0) and the concentration was adjusted to 10 µg/ml. RNAse was stored at -20°C until use.

Cloning of pln EF locus in E.coli

PCR amplification of plantaricin EF locus using plnEF2310-F- AGAGCACTATTAGGTAGTAAATAGCTGTGA and plnEF2310R- AAATAACATCATACAAGGGGGATTATTTA (Mustopa, 2013). PCR was run with *platinum taq* polymerase (invitrogen) under following condition: 94ºC for 3 min, followed by 35 cycles of denaturation at 94ºC for 1 min, annealing at 58.5ºC for 1 min, and extension at 72 ºC for 90 sec, and final extension at 72°C for 6 min.

The PCR product was purified and then ligated into vector plasmid pGEMT-easy (Promega) then transformed into *E. coli* DH5α (Novagen, USA) using $CaCl_2$-method (Sambrook and Russel, 2001). The transformants were selected on LB plates with ampicillin (100 µg/mL), X-Gal (50 µg/mL) and IPTG (100 µg/mL) at 37°C. Confirmation of white colonies containing pGEMT-plnEF (plnEF fragment) plasmid with inserts in the correct orientation were performed by PCR amplification of DNA from single *E. coli* colonies plnEF2310.

Confirmation of the correct nucleotide sequence of inserts plnEF was performed by nucleotide sequencing of plasmid recombinant at the Sequencing Service (Macrogen, South Korea). Sequence identity were examined by comparing the obtained sequences with those in the DNA databases *(http://www.ncbi.nlm.nih.gov/BLAST)*. Furthermore, Nucleotides and peptides alignment was done using Biological sequence alligment editor software *(http://www.mbio.ncsu.edu/bioedit/bioedit.html)*. *Lactobacillus* strains used as reference are accession number of FJ809773.1, JQ933106.1, JQ900767.1, HQ651181.2, X94434.2, DQ340868.2, AF522077.2, AL935253.1, and GU584091.

Cloning and constructions of plnE gene and plnF gene and transformation

The construction of either mature peptide of pln E (plnES34BamHI_F- GACTGGATCCTTTAATCGGGGCGGTTA and plnES34HindIII_R- GATCAAGCTTTTAACGAATACTTTTCAAAA or mature peptide of pln F (plnFS34BamHI_F- GACTGGATCCATGAAAAAATTTCTAGTTTT and plnFS34BHin_R- GATCAAGCTTTCCGTGGATGAATCCTCGGA) in the expression plasmid used the forward primers of each primer gene set with addition of BamHI restriction site and reverse primers with addition of HindIII restriction site.

The PCR cycle performed as follows: initial denaturation of DNA at 94 °C for 3 min, an amplification programme consisting of 35 cycles at 94 °C for 1 min, 63 °C (plnF) or 55 °C (plnE) for 1 min and 72 °C for 30 sec and the final extension of 1 cycle at 72 °C for 6 min. PCR products were separated by gel electrophoresis on a 2 % agarose gel and visualizied using UV-light illuminator. The appropriate DNA bands were excised from gel and purified with a gel extraction kit (Qiagen, Hilbert, Germany) and digested with BamHI and HindIII restriction endonucleases. The resulted gene fragments were purified and ligated into pET32a (+) vector (Novagen, USA). The fragment of either plnE or plnF gene was cloned into pET32a plasmid in-frame with thioredoxin-(His)6tag (pET32a-plnF or plnE). Ligation mixtures were transformed into E. coli T0P 10 (Novagen, USA) competent cells, using standard procedure as described by Sambrook *et al*., 2001. The transformation process will be described in the following section.

Bacterial Transformations

E. coli BL21 (DE3) pLysS cells (Novagen, USA) and competent made was using the $CaCl_2$-method (Sambrook and Russel, 2001). Briefly, the cells were grown on LB medium to an OD_{600} of about 0.5 and the culture was then cooled on ice for 10 min. Recovering of the cell was done by centrifugation at 7,000 g for 10 min. The cells were washed with ice cold $MgCl_2$-$CaCl_2$ (80 mM $MgCl_2$, 20 mM $CaCl_2$) and finally resuspended in ice cold 0.1 M $CaCl_2$. After 45 min on ice, cells were ready for transformation. Plasmids and cells were incubated for 30 min on ice, 90s at 42°C, 2 min on ice and then 45 min at 37°C in 1 mL LB medium before poured out on agar plates with appropriate antibiotics.

Expression Recombinant Plantaricin

Both of pET32(a) expression vector-subcolned plnE and pln F genes were separately expressed in *E. coli* BL21 (DE3) pLysS. Recombinant clones were selected by the addition of 100 µg of ampicillin per ml to the medium. As a negative control, *E. coli* BL21 (DE3) pLysS was transformed with pET32a (+) alone to generate E. coli BL21 (DE3) pLysS (pET32a no insert). An overnight culture of *E. coli* BL21 (DE3) pLysS (pET32a-plnF or plnE) was inoculated into 200 ml LB broth in the presence of 100 µg/ml ampicillin and was incubated at 37 °C until OD_{600} of 0.4 value was achieved. Isopropyl-β-D-thiogalactopyranoside (IPTG) was then added to the culture at a final concentration of 0,5 mM, and then, the cells were incubated further for 5 h at 22°C in broth medium at 150 rpm. The cells were harvested by centrifugation at 8,000 g at 4°C for 10 min and were resuspended in lysis buffer pH 8 containing 20 mM Tris-HCl, 500 mM NaCl and 20 mM imidazole directed to sonication (0.5 cycles of 30 output, 15 s pulse on and 60 s pulse off). The lysate was sentrifuged at 18000 g, 4°C for 30 min. After that, the supernatant was collected and the pellet resuspended in the same lysis buffer. Pellet and supernatan fraction were separated by SDS polyacrylamide gel electrophoresis 12% (SDS PAGE).

Purification of recombinant mature peptide of

PlnE and plnF

The purification method was done according to a previous study by Utama *et al.* (2000) and Li *et al*. (2010) with modification. Supernatant fraction was resuspended with resin Co^{2+} (TALON) and then binding on a rotator for 3 hours at 4ºC. After that, the sample was centrifuged at 7000 g, 4°C for 7 min. The resulting supernatant was discarded, and the resin resuspended in wash buffer (Tris/HCl 20 mM pH 8, 500 mM NaCl, 60 mM imidazole), and subsequently centrifuged at 7000 g, 4°C for 5 min. The washing process was repeated 2 times. Then resin was resuspended with elution buffer (Tris/HCl 20 mM pH 8, 500 mM NaCl, 250 mM imidazole) and binding on rotator at 4ºC for 12 h, then subsequently centrifuged at 7000 g, 4°C for 5 min. The supernatant was taken and then dialyzed using buffer (500 mM Tris/HCl, pH 8,10 mM $CaCl_2$, 1% Tween-20 (v/v)).

Protein analysis using SDS-PAGE

Tricine-SDS-PAGE method was used for determining the potein molecular weight of the gene product (Schagger and Jagow, 1987). The diluted samples (1:1 by 2× sample buffer) were loaded onto tricine-SDS-PAGE. Protein bands were visualized by staining with Coomassie Brillian Blue solution.

Table 1. Sequence identity of the plantaricin EF of *Lactobacillus plantarum* S34 based on pln EF loci

No.	Species	Identity (%)	Accession Number
1.	*L. plantarum* V90	99	FJ809773.1
2.	*L. plantarum* YM 4-3	99	JQ933106.1
3.	*L. plantarum* YM 5-2	99	JQ900767.1
4.	*L. plantarum* SP-A3	99	HQ651181.2
5.	*L. plantarum* C11	99	X94434.2
6.	*L. plantarum* J51	99	DQ340868.2
7.	*L. plantarum* NC8	99	AF522077.2
8.	*L. plantarum* WCFS1	99	AL935253.1
9	*L. plantarum* PCS20	96	GU584091

Results

Characterization of L. plantarum *S34 pln EF loci*

In an attempt to determine whether the *L. plantarum* S34 genome carried plnEF gene, PCR analysis using set of primers targeted for pln EF loci was used. The PCR results showed that the fragment with 530 bp of plnEF gene was successfully obtained by this spesific primer and individual plnE gen was shown by 102 bp band and pln F was 105 bp band (Figure 1A).

The nucleotide sequence of the plnEF loci obtained from *L. plantarum* S34 had 99% homology with those the other plantaricin EF of *L. plantarum* strains such as V90, YM 4-3, C11, J23, J51 and WCFS1 (Table 1). Based on gene components that composed loci of plnEF, plnE gene was only differed by one nucleotide at position 97 (adenine > cytidine) and there were two nucleotides substitution found in plnF gene at position 40 (guanine > timidine) and position 124 (guanine > adenine) when compared to these other strains.

The peptide similarity of the plnE and plnF from *L. plantarum* S34 had 98,21% and 96,51% with those the other plantaricin E and F of *L. plantarum* strains such as C11, NC8, WCFS1, J23, J51 and V90.

Based on peptide similarity results, plnE peptide had 98.21% similarity with all those plnE peptides producing *Lactobacillus* strain references and to be higlighted plnE in our study had one amino acid subtitution at position 33 which was C instead of K (Figure 2C). Furthermore, plnF had 96.15% similarity with almost *L.plantrarum* strains aligned which was differed by two amino acid subtitusion at position 14 (A>S) and position 42 (V>I), yet only with one Lactobacillus strain that pln F was 100% identic with (Figure 2D). The nucleotides substitution

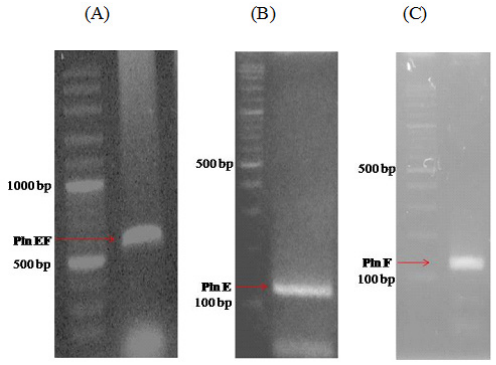

Figure 1. The agarose gel electrophoresis results of *L.plantarum* S34-derived plantaricin PCR products. (A) Locus of plantaricin EF gene with band length of 530 bp. (B) Mature peptide fragment of plnE gene with band length of 102 bp. (C) Mature peptide fragment of plnF gene with band length of 105 bp.

that found in either plnE or plnF gene did not occur at nucleotide positions (GxxxG motif) that holded important part on biological activity of this gene expression product.

To clone and expressed the individual pln gene in pET32a pression vector we used two different pair of primers to amplified either mature fragment of plnE or plnF gene which were inserted in pGEM-plnEF vector. As shown in figure 1B and C both mature plnE and plnF of fragment genes were separately succesful amplified with length of approximatly 102 bp for plnE gene and 105 bp for plnF gene.

Expression of recombinant plantaricin

PCR-amplified genes were separately subcloned into pET32a vector fused with N-terminal Thioredoxin(His)6tag as fusion parther protein generating recombinant pET32a-plnE or pET32a-plnF expressed in *E. coli* BL21 (DE3) pLysS. Sequence analysis of inserted genes in pET32a showing that only recombinant mature peptide of plnE has risen an nucleotide change at base position of 28 represented Q to K amino acid substitution at position 10 (Figure 3).

The plantaricin genes were under the control of T7 promoter and as shown in figure 4 both of recombinant plantaricins expressed in heterolougous cell produced targeted recombinant protein with yield was 0,67 mg/ml for recombinant plnE and was 0,54 mg/ml for recombinant plnF with nearly the same of molecular weight (±21 kDa) as fusion protein using the same expression vector, while no targeted protein was expressed in recombinant *E.coli* containing only pET32a with no plantaricin gene inserted after IPTG induction (Figure 3B line 2), indicating that Pln E and F were successfully expressed in E. coli as fusion recombinant protein using pET32a expression vector.

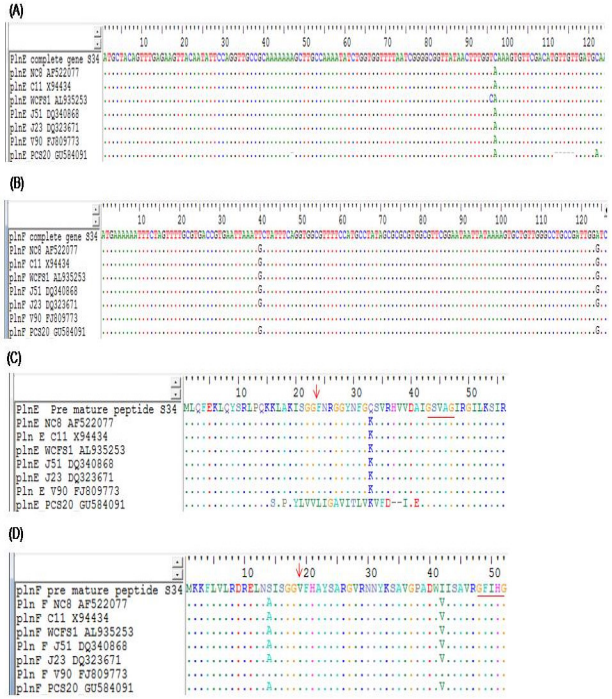

Figure 2. Alignment result of plnEF gene and their peptides isolated from *L.plantarum* S34 compared to *lactobacillus* references strains. (A) and (B) were nucleotides alignment of pre-mature fragment of plnE and plnF (C) and (D) were amino acids alignment of pre-mature fragment of plnE and plnF. The cleavage site of leader peptides at both pln E and F peptides was pointed by red narrow; the GxxxG motif was marked by red line.

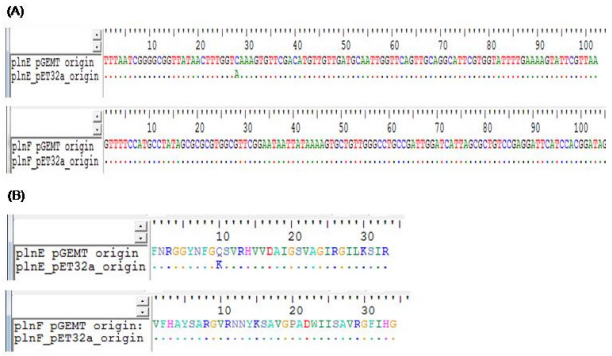

Figure 3. DNA and peptides alignment of *L.plantarum* S34 recombinant plnE and F. (A) DNA alignment of recombinant plnE or plnF retrived from pGEMT and pet32a (B) peptide alignment of recombinant plnE or plnF retrived from pGEMT and pet32a.

Discussion

In the present work, we could retrieve plnEF gene from operon plnEFI from Indonesia traditonal fermented meat (Bekasam)-isolated *Lactobacillus plantarum* S34 strain origined from Lampung province. The exact length of intact operon of plnEFI loci, that was composed of plnE gene, plnF gene, plnI gene and its native promoter, in *L. plantarum* S34' genome could not be fully obtained due to the limitation of primer pair used. Based on plnEFI loci analysis from the other *Lactobacillus* strains showing that this operon were almost the same in their nucleotides length (Diep *et al*., 1996; Kleerebezem *et al*., 2003; Maldonado *et al*., 2003; Navarro *et al*., 2008; Tsapieva *et al.*, 2011). PlnEFI loci of *L. plantarum* WCFS1 (human saliva origin), NC8 (grass silage origin), JC51(Rioja red wine origin), C11 (vegetable origin), SP-A3 (GI tract), and V9 has 1225 bp length, and only *L. plantarum* PC20 (home made cheese origin) known so far has plnEFI loci (1219 bp) shorter than the other strains due to mutation event in the plnE gene (Cho *et al.*, 2010). It seems that the environment niches where LAB originated from could significantly influence the genetic stability of strains in adaptive evolution for survival under stress conditions (Machielsen *et al*., 2010). *L. plantarum* S34-originated partial operon of plnEFI loci also exhibited diversity in plnE and F nucleotides composition (Figure 3) as consequence of environmental presure with in *Bekasam.* Furthermore, mutations on plnE and F genes in our study was not occur in GxxxG motifs and may not impact on bactericidal activity of plantarcin produced. Fimland *et al.* (2008) stated that the antibacterial mechanisme of plnE and plnF peptides exclusively involved complementary action through GxxxG motifs interaction of both peptides. Broadly bacterial kiling properties exhibited by *L. plantarum* in fact that these species have been equipped with natural ability to produce extracellulary bacteriocins such as plnEF, plnJK, plnN, orf3/4, plnC8αβ, and orfZ2 (Anderssen *et al*., 1998; Maldonado *et al*., 2003; Navarro *et al*., 2008; Diep *et al.*, 2009).

Plantaricin are ribosomally synthesized extracellular short peptides produced by many *L. plantarum* species. These of class II of bacteriocins have been an atractive natural antibacterial agents to replace chemical additive agent in food preservatives in recent years. Many reports have proven the ability of *L. plantarum* species to produce plantaricins that surprisingly exhibits not only diversity in their biochemical properties but also in their biological properties especially in bacterial killing ability spectrum against gram-positive and negative bacteria, food-borne pathogenic and spoilage bacteria (Klaenhammer, 1993; Atrih *et al*., 2001; Van Reenen *et al*., 2003; Maldonado *et al*., 2003; Muller *et al*., 2009; Hata *et al*., 2010). In our previous study, *L. plantarum* S34 had been successful isolated from fermented meat *(bekasam)* and its identity was confirmed by 16s RNA sequencing (Mustopa, 2010). Furthermore, plantaricin-S34 produced by *L.*

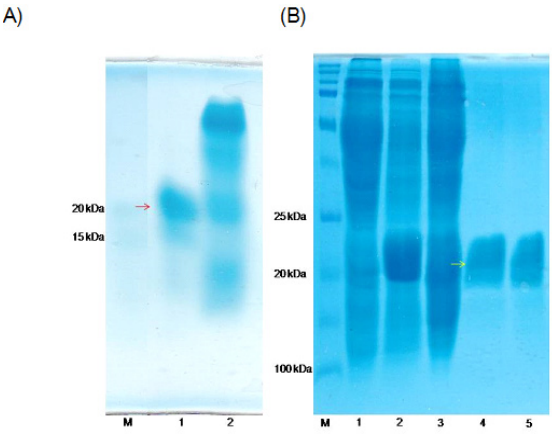

Figure 4. SDS-PAGE of the fused recombinant mature peptide of PlnE (pointed by red arrow) and plnF (pointed by yellow arrow) with molecular weight of ±21 kDa from recombinant *E.coli* cultured at 22°C, 5 hours incubation and 0.5 mM of IPTG (A) Line 1 was soluble fraction of pET32a-plnE ; Lane 2 was elution fraction of lane 1 after TALON-treated supernatant fraction (B) Lane 1 was soluble fraction of pET32 with no plnF insert; Lane 2 was soluble fraction of lane 1 under IPTG induction; lane 3 was pET32-plnF after induced by IPTG; lane 4 and 5 were elution fraction of TALON-treated supernatant fraction before and after dyalisis treatment. M was protein standard (BioRAD).

plantarum S34 has been purified and characterized its biochemical properties and exhibited excellent antiviral activity against HCV viral helicase in vitro (Chandra, 2013).

Plantaricins particularly those that belong to class II bacteriocins received considerable attention as food preservatives and as potential replacement of antibiotics (Todorov *et al*., 2007; Todorov *et al*., 2009). The conventional procedures to collect native plantaricin peptides directly from *L. plantarum* have recently been established with excellent biological activity and high in protein purity obtained (Atrih *et al.*, 2001; Gong *et al*., 2010; Hata *et al.*, 2010; Zhu *et al.*, 2014), yet those procedures is hard to be applied to produce high yield of native plantaricins directly from natural producer (Tiwari and Srivastava, 2008a). In this study, we have established an operon construction and expression of fermented food-origin mature peptide of plnE and plnF isolated from *L.plantarum* S34 in heterologous *E.coli* strain BL21 (DE3) pLysS to increase plantaricins yield as recombinant fusion with thioredoxin-(His)6tag using optimized conditon (0.5 mM IPTG, temperature induction was 22°C and incubation time was 5 hours). With this optimized condition fairly high recombinant plantaricins was obtained. Native bacteriocins (LR14α, LR14β, and LR14αβ) isolated from *L. plantarum* LR14 using three steps of purification could only retrieve 0,002 mg/ml to 0,003 mg/ml of pure bacteriocins (Tiwari and Srivastava, 2008a). Meanwhile, our recombinant plantaricins yield was approximatly still half of yield of *L. plantarum* S34-originated the unpured native bacteriocins (1,05 mg/ml) from partially purification using ammonium sulfate method (Mustopa, 2013), yet we predicted that this yield of native bacteriocins would be far less than those heterologous cells-isolated recombinant bacteriocins if further steps of purification applied. Unpredicted result came from sequence result of mature fragment of plnE after cloned in pET32a and heterologously expressed in *E.coli* BL21 (DE3) pLysS was found. There was emerged nucleotide substitution that changed glutamine to lysine at position 10 of peptide, we assumed the spontaneous mutation occured in recombinant mature peptide of plnE due to reading error (for example lack of 3'-->5' proofreading exonuclease activity) of Taq polymerase used (Eckert and Kunkel, 1991). In our point of view, although this substitution of amino acid may not impact directly on recombinant proteins yield but with improved methodology applied in our experiment we have succesfully increased the plantaricin production when compared to recombinant plantaricin E and F yield reported by Fimland *et al.* (2008).

The application of heterologous protein epression technology for increasing of the class 2 bacteriocin yield have been proven (Mccormick *et al.*, 1998; Richard *et al*., 2004; Klocke *et al*., 2005; Fimland *et al.*, 2008; Rogne *et al*., 2009; Basanta *et al.*, 2010; Fang *et al*., 2010; Borrero *et al*., 2012).

Conclusion

In summary, partial operon of plnEFI loci isolated from *L.plantarum* S34 has been characterizied and their respective genes, plnE and plnF, then cloned *E.coli* BL21(DE3) pLysS produced as recombinant peptides for large scale production and for their application in various field of biotechnology and medicine.

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