Title, Times New Roman 14 pt, bold, Align text center, Initial cap, Ex. Construction and Expression of Recombinant Bacteriocin from *Bacillus subtilis*

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ABSTRACT

At present, the problems encountered with antibiotic-resistant bacteria are increasing. So, the scientists are looking for natural substances to prevent these problems. The substance used is a bacteriocin produced by one bacterium to eliminate other bacteria to protect itself. In this research, we examined bacteriocin produced by *Bacillus subtilis*, by searching the bacteriocin database. A novel bacteriocin named LCI46 which is 195 base pair was designed and expressed in *Escherichia coli* BL21 production system. To our knowledge, no study has been conducted on this recombinant bacteriocin before. After preliminary experiment LCI46 displayed better expression and antimicrobial potential. Thus, the bacteriocin LCI46 was produced and purified. Tricine SDS-PAGE analysis of the purified bacteriocin showed a single band of peptide with molecular weight of 6.05 kDa. The highest expression was observed after 4 h induction. The purity of the LCI46-Histag was determined to be higher than 90%, with a final yield of 0.5 mg/l of LB broth. The recombinant LCI46 showed antibacterial activity against Gram positive (*Staphylococcus aureus* and *Micrococcus luteus*) bacteria with minimum inhibitory concentration (MICs) of 100 µg/ml and 50 µg/ml, respectively. Future studies should be performed to investigate other mechanism of actions of the LCI46. (Times New Roman 12 pt, regular, Justify, not exceeding 250 words)

Keywords: Antimicrobial, *Bacillus* sp., Bacteriocin, Foodborne pathogens, Microbial. (3-5 words, Times New Roman 12 pt, Align text left, Justify, Alphabetically, First word capital only)

INTRODUCTION

Food industry depends on chemicals for the preservation and to increase the shelf life of foodstuff. The use of chemical preservatives may cause adverse effect on human health (Zubeir and Owni, 2009). Thus, there is a need to search new natural preservatives for the preservation of food. The advantages of using antimicrobial peptides is that it preserves the food without changing quality and are not harmful (Wang et al., 2016). The current problem of food-spoilage, food products can be preserved by using microbes and their antimicrobial products such as Nisin bacteriocin, which improve the shelf-life of food and enhance the food safety (Galvez et al., 2014 and Song et al., 2014).

Bacteriocins have been defined as proteins or protein complexes which are bactericidal against other bacteria in the environment (Tagg et al., 1976). The concept was widened to include peptides of a broad inhibitory spectrum (Perez et al., 2014). LAB bacteriocins have been recently classified into three classes (Klaenhammer et al., 1993): I, lantibiotics; II, small heat-stable unmodified peptides; and III, large heat-labile proteins. Classes I and II comprise the most abundant and best studied bacteriocins. The diversity of class II bacteriocins has motivated its division into three sub-groups: IIa, bacteriocins with antilisterial effect; IIb, two-peptide bacteriocins; and IIc, sec-dependent secreted bacteriocins.

The antimicrobial peptide LCI was first screened and isolated from a *Bacillus subtilis* strain named A014 against the Gram-negative pathogen *Xanthomonas campestris pv Oryzea* (Liu et at., 1990). This bacterial pathogen has effect on rice and result in leaf-blight disease, which is a serious threat to rice production and causes great losses in yields. Until now, there has been no efficient method for controlling this disease. In addition, LCI also has antagonistic activity against Gram-negative bacterium *Pseudomonas solanacearum* PE1, but it does not inhibit *Erwinia carotovora* sub sp. *Carotovora* or *Escherichia coli*.

Recombinant DNA technology is considered as a cost-effective and scalable method of producing significant amounts of active bacteriocin (Liu et al., 1990 and Xi et al., 2013). Unfortunately, there is no paper reported so far on the recombinant expression of active

bacteriocin form NCBI genbank. The expression of recombinant peptides in *Escherichia coli* expression systems have been well established and was used (Xi et al, 2013 and Wang et al., 2008).

In this study we report the construction, expression, purification and antimicrobial activity of the novel active bacteriocin from NCBI genbank. The recombinant bacteriocin (LCI46) was expressed with an N-terminal 6×His tag for effective purification. The determination of the antibacterial activity of the *E. coli* expressed recombinant bacteriocin is in support of its potential application as active antimicrobial agent. This work is under pending petty patent.

MATERIAL AND METHODS

Materials

Staphylococcus aureus (ATCC 6538) and *Micrococcus luteus* (TISTR 745), were kindly donated by Department of Microbiology, King Mongkut's University of Technology Thonburi University Thailand. Luria-Bertani agar (LB agar) (10 g/l tryptone, 10 g/l NaCl, 5 g/l yeast extract 12 g/l agar and antibiotic) was used as selective medium for the expression in *E. coli* and testing of all the strains.

Construction of the expression vectors pET 25b (+)-LCI46

Briefly, the amino acid sequence of LCI46, which contains 46 amino acids was obtained from the antimicrobial peptide database (https://www.ncbi.nlm.nih.gov/genbank/). To construct the bacterial expression vector for the production of the recombinant bacteriocin, a nucleotide sequence composed of 18 bp 6×His tag coding sequence, a bacteriocin sequence and a termination codon were synthesized by U2bio (Seoul, South Korea). To facilitate the upcoming clone, this nucleotide sequence was incorporated with the *Nde*I and *Bam*HI restriction sites during the nucleotide synthesis, and was inserted into the intermediate vector pBHA (Seoul, South Korea).

After double digested with *Nde*I and *Bam*HI, the DNA fragment was cloned into the *E. coli* expression vector pET25 b (+) (Invirogen, Carlsbad, CA, USA), to obtain pET25b (+)-LCI46. *E. coli* host strain BL21 (DE3) pLysS (Invitrogen, Carlsbad, CA, USA) was used for expression.

Bacterial transformation, expression and purification of recombinant LCI

E. coli BL21 (DE3) pLysS competent cells (Invitrogen, Carlsbad, CA, USA) were transformed with pET25b (+)-LCI46 by a heat-shock method (Karikari et al., 2017). IPTG-induced recombinant bacteriocin expression was performed as described previously (Yang et al, 2017). Transformant was grown in 50 ml of LB medium at 37 ºC, in an incubator shaker at 200 rpm, for 4 h, and then we induce with IPTG to the final concentration of 1 mM until (OD600) reached 0.8 to 1.5. The cell pellet was harvested by centrifugation at 10,000 ×g for 10 min and resuspended in 10 ml of Tri-HCl buffer (pH 7.4). Cells were sonicated with sonic wave to extract protein. Then, the recombinant bacteriocin was subjected to 16.5% tricine SDS-PAGE. The concentration of the LCI46 bacteriocin was determined by protein constant in the supernatant (Bardford protein assay) at a wave length of 595 nm and compared with standard protein BSA (bovine serum albumin) (Sambrook and Russell, 2001).

The purification procedure was performed by Ni^{2+}affinity chromatography (HisTrap™, ÄKTA, GE Healthcare). Briefly, the bacteriocin sample was passed through a column and extensively washed with binding buffer (100 mM Tris base, 500 mM NaCl, and 20 mM Imidazole, pH = 7). And then, elution buffer (20 mM Tris, 500 mMNaCl, and 500 mM imidazole, pH = 7.0) were applied to elute the bacteriocin. The purified bacteriocin were dialyzed overnight at 4 °C by a 3 kDa cutoff dialysis membrane. The eluted fractions were collected for further analysis by tricine SDS-PAGE.

Antimicrobial assays

To determine whether antimicrobial compound was released into culture supernatant, we confirmed by testing the antimicrobial activity using the well diffusion assays, as described by Begley et al. (Begley et al., 2009). The diameter of the wells used for the assay were 6 mm. The purified LCI46 was tested against all the indicator strains: *Staphylococcus aureus*, *Micrococcus luteus* in order to determine the spectra of inhibition. All indicator strains were used at an inoculum of optical density (O.D.600) 0.2 to 0.3. Plates were incubated for 20 h at 37 ºC and examined for zones of inhibition.

The MICs of both the *Staphylococcus aureus* and *Micrococcus luteus* bacterial strains were also determined using a microtiter broth dilution method (Tian et al., 2009 and Domeneghetti et al 2015). Each

strain was incubated to a mid-log phase 2-7×10^5 CFU/ml optical density (OD600) 0.2 to 0.3 in LB medium at 37 ºC. The purified LCI was serially diluted before being added to each well of a 96-well plate. In brief, 100 µl cell suspension and 20 µl of purified LCI of different concentrations were added to each well and incubated at 37 ºC for 16-20 h with vigorous agitation. Ampicillin (100 µg/ml) and LB medium were used as positive and negative controls, respectively. Bacterial growth was evaluated by measuring the absorbance of the bacterial culture at 600 nm using a Synergy H/F multi-mode microplate reader (NS-100 model, Hercuvan, United Kingdom). MIC was defined as the minimum concentration of purified LCI that caused 100% inhibition of bacteria growth. All assays were carried out in triplicate.

RESULTS

Gene cloning and sequence determination

As per the genome annotation of *Bacillus subtilis*, the LCI Bacteriocin from the bacteria have the theoretical pI value per molecular weight (pI/Mw) of 8.65/6031.74864 Dalton. BLASTP analysis showed it had high identities (93.3%) with identified LCI bacteriocin template in the Database. Signal sequence prediction demonstrated that the LCI46 bacteriocin of *B. subtills* had signal peptides. The supposed cleavage point of the signal sequence was after AA (amino acid) 19. The signal sequence will influence the soluble property of the expressed protein in *E. coli* host. So, the gene of the signal sequence of the LCI46 was deleted, then linked in pET-25 b (+) vector. On the contrary, the codon usage tendency may also affect the expression level of the target protein. Thus, we chose *E. coli* BL21 as the host for protein expression.

Construction of expression vector

pET-25 b (+) expression vector contains restriction sites for homologous recombination to facilitate the process of integration in *E. coli* genome. LCI46 gene was amplified by PCR (polymerase chain reaction). Double digestion was performed before the ligation method was used. The results of the double digestion showed that there was successful insertion of the target fragments into the vector. Additionally, the DNA sequencing used to confirm that there was successful construction (Data not shown).

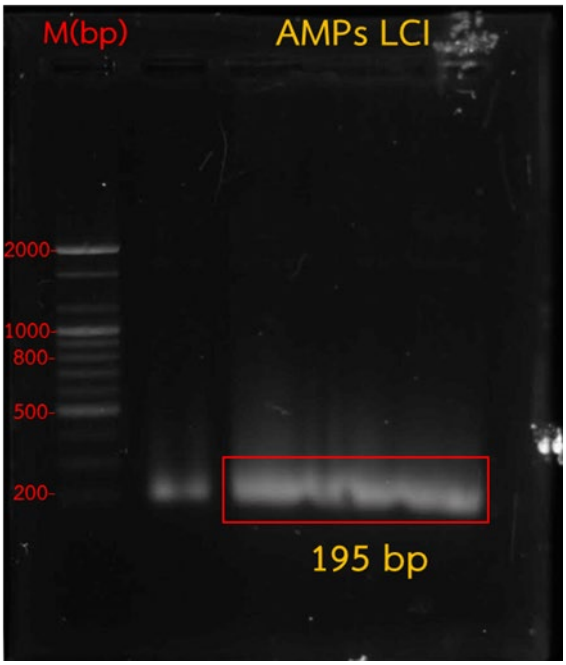

Figure 1. Amplified PCR product of LCI46 gene free of target gene displayed a 195bp band.

Expression and purification

The recombinant LCI46 bacteriocin was purified by affinity purification using the Ni^{2+} column. Tricine SDS-PAGE results showed a single band of peptide with a molecular weight of approximate 6 KDa (Fig. 2), indicating that the LCI46 was efficiently eluted by 500 mM imidazole. Overall, a final yield of approximate 0.5 mg purified LCI46 of 90 % purity was obtained from 1L of cell culture medium.

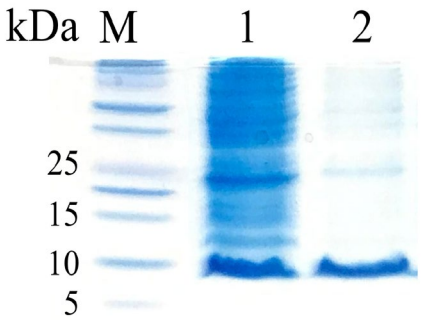

Figure 2. Purification of recombinant LCI46 bacteriocin on 16.5% Tricine SDS-PAGE analysis. M: marker; lane 1: supernatant; lane 2: elution LCI46.

Antimicrobial activity

Next, the antibacterial activity of the LCI46 was tested on bacterial pathogens including *S. aureus* and *M. luteus*. Ampicillin at a concentration of 100 µg/ml was used as a standard antibacterial agent. As determined by inhibition zone assays, treatment with the purified LCI46 at a concentration of 100 µg/ml resulted in obvious occurrence of inhibition zones on both the tested strains of M. *luteus* and *S. aureus*. The data presented in Table 1, shows that the recombinant LCI46 displayed antibacterial activity. The MIC values of the bacteriocin against only Gram-positive *M. luteus* and *S. aureus* were determined to be 50 and 100 µg/ml respectively. The results of the agar disk diffusion also confirmed the antibacterial activity of recombinant LCI46 by the distinct inhibition zones that appeared around disks treated with the peptide. The diameter of the zone of inhibition for *S. aureus*, *M. luteus* were 4 and 10 mm, which was comparable to that of the Ampicillin as the control positive (Fig. 3).

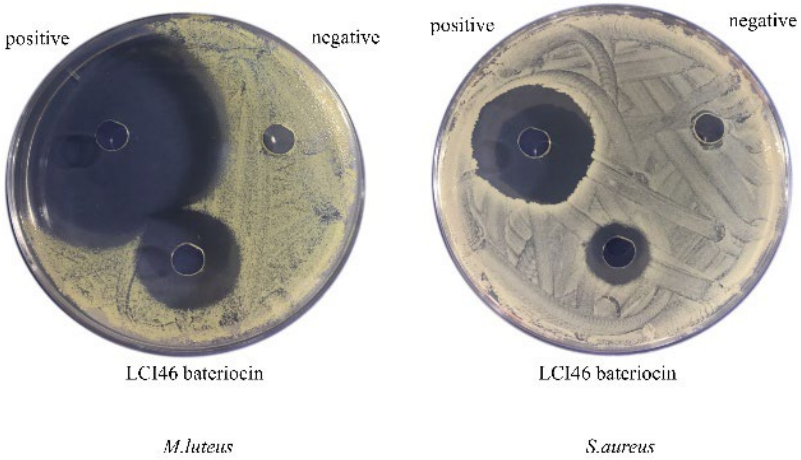

Figure 3. Well diffusion assay of the LCI46 performed on *M. luteus* and *S. aureus*. Positive: indicates ampicillin (100 µg/ml); Negative: represents the bacteriocin buffer alone; LCI46 bacteriocin; bacteriocin sample after purification (100 µg/ml).

Table 1. Microdilution method to determine the concentration to inhibit bacteria

Bacterial strain	Diluting concentrations of LCI46 bacteriocin (µg/ml)						MIC[a] (µg/ml)
	100	75	50	25	12.5	6.25	
M. luteus	-	-	-	+	+	+	50
S. aureus	-	+	+	+	+	+	100
Negative control	+	+	+	+	+	+	/

Negative control: bacteriocin buffer.
+: observed bacterial growth.
-: no bacterial growth.
/: no inhibition.
[a]: MIC: minimal inhibitory concentration of the bacteriocin.

DISCUSSION

LCI46 bacteriocin is part of protein found in *Bacillus* bacteria, and it plays a role in the inhibition of related species. Previously, similar bacteriocin from *Bacillus subtilis* such as LCI47 has only been chemically synthesized and characterized (Saikai et al., 2019). Nonetheless, the isolation of LCI46 bacteriocin from other organisms or its chemical synthesis can be a complicated and expensive practice; in addition, the yield of synthesis cannot be insured. Therefore, it seems that the proposed pathway toward industrialized production of LCI46 bacteriocin is its recombinant expression systems have some evident benefits as the nature of the bacteriocin properties of this peptides means are potentially deadly to host cell or change the production yield (Liu et at., 1990).

Furthermore, the small size property of bacteriocin make them highly susceptible to proteolytic degradation. The active bacteriocin was immediately used as a test.

CONCLUSION

In summary, the findings obtained in the current study showed that unknown gene from genbank is able to manage and improve the stability of the production system on *E. coli.* Recombinant LCI46 bacteriocin displayed highest antibacterial activity against Gram positive bacteria. However, the results of the present study may also be helpful for further study. The method could be used for investigation towards industrialized production of other bacteriocin in the genbank.

ACKNOWLEDGEMENTS

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