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Expression and Purification of Nisin in E*scherichia coli*

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ABSTRACT

Fusion expression is a promising strategy for the production bioactive peptides in *Escherichia coli* to enhance either soluble protein level or purification potential. Nisin is the bacteriocin that had been extensively studied and had been widely applied in many areas such as food, pharmaceutical. However, scientific reports on recombinant nisin production in *E. coli* are still insufficient. In this study, we constructed a new expression plasmid containing the coding sequence of NusA, hexahistidine and *Lactobacillus lactic* nisin coding sequence. Next, we introduced the expression plasmid into BL21 *E. coli* and produced the recombinant fusion nisin in *E. coli*. Recombinant *E. coli* extract was purified by nickel affinity chromatography and resulted in 77% yield with 55% purity. The bioactive nisin was successfully released from NusA-6xHis-Nisin fusion protein by the endonuclease. The nisin showed its antibacterial activity on *Listeria monocytogenes* with activity unit of 18.9 AU/mg. The nisin bioactivity is stable at the temperature range of 30-90^{o}C and in pH range of 1-12. The results showed that the new construction was appropriate for production of nisin bioactive peptides.

Key-words: Nisin, Recombinant protein, *E. coli*, Expression system, Bacteriocin

INTRODUCTION

Nisin is a bacteriocin which was well known and widely used in many types of applications. Up to now, seven natural Nisin variants (A, Z, F, Q, U, U2, H) have been recognized [1]. Nisin A and Z were the most variant nisins in nature. Sequences of nisin A and Z was differed in only one amino acid residue at position 27 (nisin Z contains asparagine,nisin Acontains histidine); meanwhile, nisin Q and nisin A was differed in four residues [2]. These nisins shared similar antibacterial spectrum. Besides, a new study showed that nisin Q can inhibit oxidation better than nisin A [3]. Nisin is a cation peptide from bacteria *Lactococcus lactis* [4-7].

Mature nisin peptide sequence has 34 amino acids, five ring structures in the molecule (A, B, C, D, E) with one lanthionine (ring A) and four β-methyllanthionine (rings B, C, D, E) [1]. Hsu *et. al.* [8], showed that two thio-ether rings at N-terminal of nisin performed an important role in interacting with lipid II which was a component involved in the formation of gram-positive bacterial peptidoglycan cell wall. Nisin had wide antibacterial spectrum against gram-positive bacteria and some gram-negative bacteria. Nisin inhibited the growth of those species by forming pores in the bacterial cell membrane or inhibited the formation of peptidoglycan wall via interacting with lipid II. Thereby, the causes of target bacterial death were cytoplasmic membrane depolarization, ion exchange disorder, cell energy production decreasing [5,9-11]. Furthermore, antibacterial activity of nisin was stable in low pH or high temperature conditions [5,12]. Accordingly, nisin has been applied in food preservation and medical purposes for almost 30 years. Nisin was approved as a safe food preservative by FAO, FDA, and was licensed to be used in more than 60 countries [13].

The applicability and safety of nisin brought huge needs of production. However, production of nisin was still

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limited. Up to now, nisin was only produced by natural nisin production strains. Precursors of nisin were synthesized as a peptide of 57 amino acids. These pre-peptide sequences need several transformation stages after the translation to form active nisin sequences with 34 amino acids. This process required 11 components involving in prepeptide (NisA), modification (NisB and NisC), secretion (NisT), processing (NisP), regulation (NisR and NisK) and immunity (NisF, NisE, NisG and NisI) [14-22]. In the face of that reality, very few successful studies of recombinant nisin production were reported. Karakas *et. al*. [23], expressed nisin A as a fusion protein with 6xHis tag to facilitate nisin collecting and purifying. In that research, a precursor nisin fused with 6xHis tag in N-terminal (prenisin-His_6) was expressed in *E. coli* and purified using nickel affinity column under denaturing condition [23]. However, results of the collection of active nisin have not been reported. With the aim of producing recombinant nisin, in this study, we constructed and expressed nisin in *E. coli* in fusing form with 6xHis and NusA tag.

MATERIALS AND METHODS

This study proceeded in July 2017 at Department of Molecular and Environmental Biotechnology, Faculty of Biology-Biotechnology, University of Science, Vietnam National University Ho Chi Minh City, Vietnam.

Nisin fusion expressing vector- Nisin coding gene (*kN*) was designed according to previously published peptide sequences (BAC145) [2,3]. Gene *kN* was amplified by PCR reaction with specific primers containing the recognition sites of the restriction enzymes XhoI at the 3' end and BamHI at the 5' end (5'BamHI-kN). Recombinant plasmid pET43.1a-*kN* was structured by cohesive cloning with two restriction enzymes XhoI and BamHI. DH5α clones containing recombinant vector was selected by ampicillin antibiotics (100µg/ml) and PCR using T7 terminator and 5' BamHI-kN primers. pET43.1a-*kN* vector was extracted and the fusion gene was analyzed by sequencing.

Inducing expression and collecting of the fusion nisin- The plasmid pET-*kN* was transformed into *E. coli* BL21 (DE3) competent cells, cultured and selected in medium containing ampicillin (100µg/ml) and PCR with primer pair 5' BamHI-kN/T7 terminator. Single bacterial colony *E. coli* BL21 (DE3)/pET-*kN* were cultured in LB medium containing ampicillin (100µg/ml), at 37°C, 250 rpm overnight. 1/20 (v/v) of the seed culture was inoculated into LB medium containing ampicillin and continuously incubated to log phase (OD600 approx. 0.8). IPTG was added at a final concentration of 0.8 mM and the mixture was cultured for an additional 4h at 37°C, 250 rpm. The bacterial cells were harvested by centrifugation (5000 rpm, 5 min) and the bacterial pellet was resuspended in lysis buffer (Na_2HPO_4 50 mM, NaCl 300 mM, Imidazole 10 mM pH 7.4). This was followed by sonication at 4°C and centrifuged (13,000 rpm, 10 min). The protein present in supernatant and pellet were tested by SDS-PAGE.

SDS-PAGE and Western blot- The protein expression purity levels were ascertained by SDS-PAGE, silver staining or Coomassie blue R250 staining. The presence of recombinant proteins was determined by Western blotting using a primary anti-6xHis-tag antibody (Invitrogen) and a secondary anti-Ig-G antibody conjugated to HRP (Invitrogen).

Nisin purification- The *E. coli* BL21 (DE3) cell extract was collected and purified by using Ni-NTA resin column (Histrap HP 1ml, GE Healthcare). Histrap HP 1 ml column was equilibrated with cell lysis solution. The supernatant was collected from cell lysis solution (Na_2HPO_4 50 mM, NaCl 300 mM, Imidazole 10 mM pH 7.4). The supernatant of cell lysis was added into column. Non-specific binding proteins were washed with 10 times column volume by followed buffers: Na_2HPO_4 50 mM, NaCl 300 mM, Imidazole 20 mM pH 7.4. Fusion nisin was eluted by these buffers: Na_2HPO_4 50 mM, NaCl 300 mM, Imidazole 100 mM pH 7.4. The purity of the protein was confirmed by SDS-PAGE, silver staining. The concentrations of purified proteins were measured using Bradford assay and stored at -30°C.

After NiNTA purification, fusion NusA-6xHis-Nisin was then dialyzed using PBS buffer (4°C, overnight). Protein dialyzed sample was concentrated using amicon 10kDa (Merck Millipore). The fusion nisin was then treated by enterokinase (Department of Molecular and Environmental Biotechnology, University of Science) at 25°C in 16hto release fusion tag.

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Evaluation of nisin bioactivity- Nisin bioactivity was determined with indicator bacteria *Listeria monocytogenes*either by agar diffusion method or polyacrylamide gel. Single bacterial colony *Listeria monocytogenes* were cultured in TSB medium at 37°C, 250 rpm overnight. 1/20 (v/v) of the seed culture was inoculated into TSB medium and continuously incubated at 37°C under shaking condition 250 rpm until cell density reaches 0.1 (OD_{600}=0.1). The cultured was then diluted with TSA 0.8% medium at1/50 (v/v). Using agar diffusion method, the TSA 0.8% diluted cultured were poured onto a TSA 1.5% gel plate. 6 mm in diameter and 3 mm in height wells were made on the surface of the gel and nisin samples were added into that wells. The diluted cultured were also poured directly onto a previously run polyacrylamide gel. The Nisin protein samples were treated with non-reducing conditions and separated by electrophoresis. The plates and gels were then incubated at 37°C for 6h and observed for fade rings formation.

The activity of nisin samples was evaluated by agar diffusion method with serial double dilution. Antibacterial activity of the samples was determined by the diameter of their inhibition zones. The highest dilution fold (n) with inhibition diameter ≥2 mm was recorded [24]. Nisin activity unit is the reciprocal using following formula:

$$\text{Activity units (AU/ml)} = 2^n \times 1000/V$$

n =Highest dilution fold, V =Test volume

$$\text{Specific activity (AU/mg)} = \frac{\text{Activity units (Au/ml)}}{\text{Target peptide concentration (mg/ml)}}$$

Stability of fusion nisin- Stability of nisin segments whose activity confirmed was tested by varying temperature and pH. Samples were incubated at 30, 50, 60, 70, 80, 90, 100°C for 15 minutes then determined for antibacterial activity against *Listeria monocytogenes* by agar diffusion method. The pH-stability of nisin was also analyzed. Different samples of nisin were subjected to different pH conditions from 1 to 12 at 4°C for 1h then determined for antibacterial activity against *Listeria monocytogenes* by agar diffusion method.

RESULTS

Construction of expression vector for nisin in *E. coli*- Nisin is naturally synthesized in *Lactococcus lactis*, which was well known AT rich organism [5,6,9]. Analysis of codon usage of *Lactococcus lactis* indicated that A and/or T ending codons are predominant in the organism [25]. For expressing nisin in *E. coli*, we analyzed nisin coding sequence to address whether the sequence was adaptable to *E. coli* codon usage. By using GenScript software, our result showed that nisin coding sequence has no tandem rare codon (the CFD, Codon usage Frequency Determination, value is 0%) in comparison to *E. coli* codon usage (Table 1). Besides, CAI (Codon Adaptation Index) value was 0.95 compared to ideal value range from 0.8-1.0. Previous research demonstrated that the base usage, codon usage and amino acid usage are changed with GC content in linear correlation [26]. Our gene analysis data showed GC content of nisin coding gene was 48.56% compared to the ideal range from 30% to 70% (Table 1). The nisin gene was adaptable and can be expressed in *E. coli* without optimization.

Table 1: Adaptation of nisin coding gene in *Escherichia coli*

Essential parameters	Actual Value	Ideal Value
CAI	0.95	0.8 – 1.0
CFD (%)	0	< 30
GC content (%)	48.56	30 – 70

CAI: codon adaptation index; CFD: Codon usage frequency determination

NusA (N utilization substance A) was a transcription termination, which has been well known as a functional sequence that helps to increase soluble protein in *E. coli* [27-29]. In the aim of expressing nisin in *E. coli* as soluble peptide, we fused nisin encoding gene with NusA at N-terminal. A 6xHis sequence was also added for purification purpose. In order to separate the NusA-6xHis peptide from nisin peptide by enterokinase, we fused an enterokinase cutting site onto the 5' end of nisin coding gene (Fig. 1A).

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The optimized nisin coding (*kN*) was inserted into a pET43.1a vector by cohesive cloning with two restriction enzymes BamHI 5' end and Xhol 3' end. pET43.1a-*kN* recombinant vector was selected and confirmed by PCR with specific primers 5' BamHI-kN and T7 terminator (Fig. 1A).

DNA sequencing results confirmed the pET43.1a-*kN* recombinant vector structure was the same as the original design; *kN* gene was cloned in-frame into pET43.1a vector (Fig. 2).

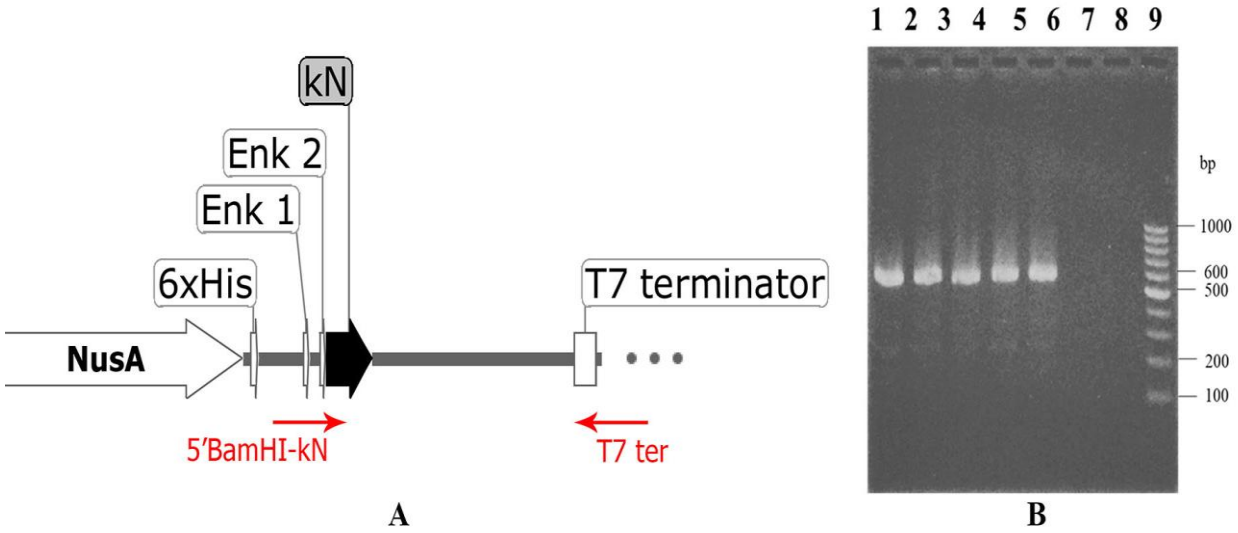

Fig. 1: Nisin expression vector construction

A: Nisin expression vector map; B: Result of selection recombination vector by PCR with 5' BamHI-kN and T7 terminator; 1-5: selective clones; 6: PCR negative control with distilled water; 7: Negative control with pET43.1a

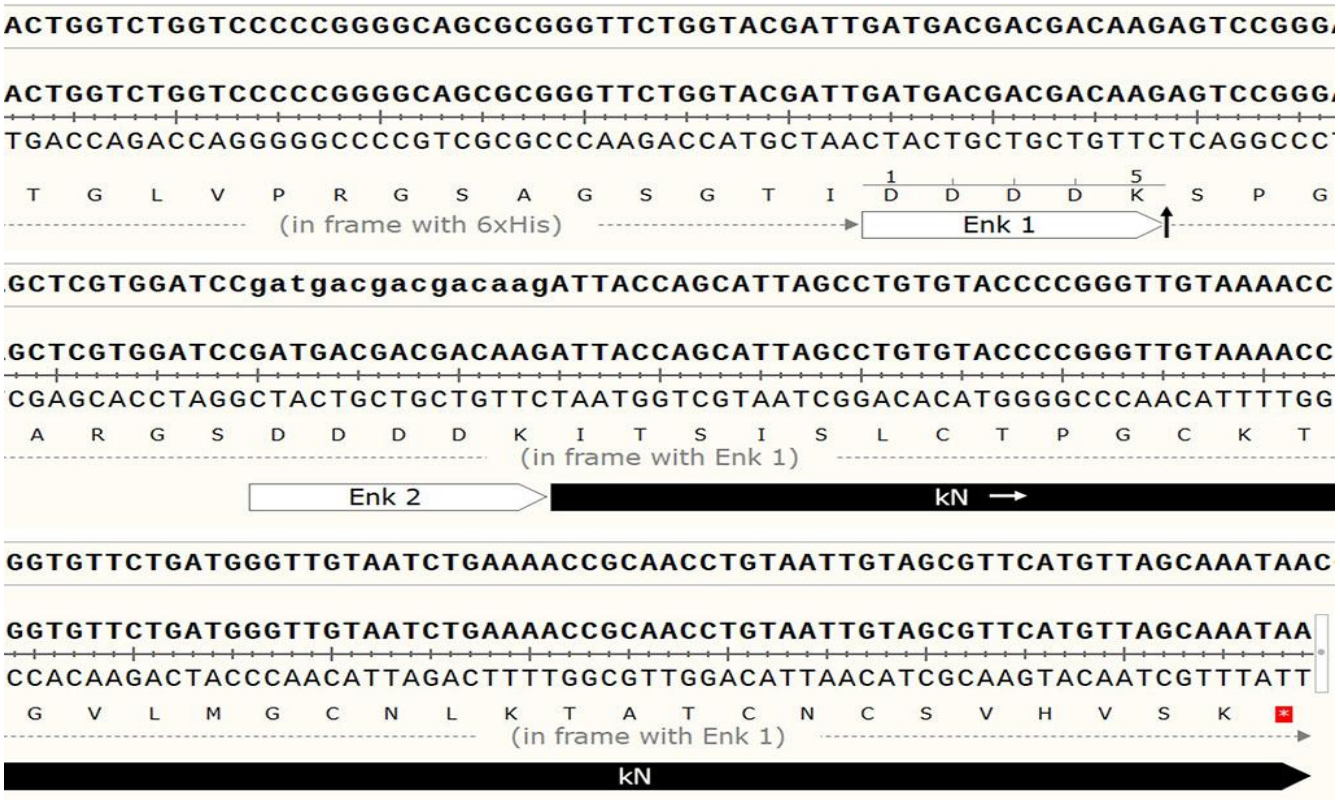

Fig. 2: pET43.1a-kN sequencing

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Expression, collection and purification of nisin- *E. coli* BL21 (DE3) was a well-known host strain for expressing recombinant protein such as: fast high-density cultivation, inactivate protease genes and containing *hsd S* (rB^- , mB^-) which help to maintain plasmid in the *E. coli* cell [30, 31]. In order to express nisin peptides in *E. coli,* the *E. coli* BL21(DE3) was used as host strain. The recombinant strain *E. coli* BL21 (DE3)/pET43.1a-*kN* then were induced by 0.8 mM IPTG and analyzed. Our data on analyzing the induced *E. coli* extract exerteda band of protein at about 66kDa as expected while that of non-induced *E. coli* BL21(DE3)/pET43.1a-*kN* and the control *E. coli* BL21(DE3)/pET43.1a did not exert the protein band (Fig. 3A). The expressed fusion protein was confirmed by WB with anti-6xHis antibody (Fig. 3B).

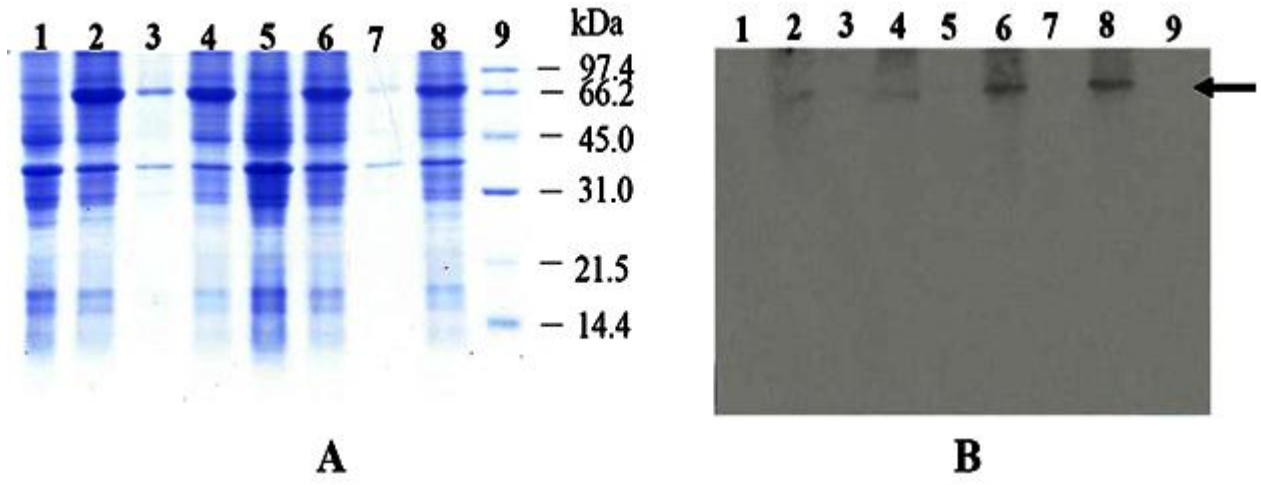

Fig. 3: NusA-6xHis-Nisin fusion protein expression in BL21 *E. coli*
A: Glycine SDS-PAGE, 1. BL21 (DE3)/pET43.1a; 2. BL21(DE3)/pET43.1a/IPTG cell disruption; 3. BL21(DE3)/pET43.1a/IPTG pellets; 4. BL21(DE3)/pET43.1a/IPTG supernatants; 5. BL21(DE3)/pET43.1a-kN; 6. BL21(DE3)/pET43.1a-kN/IPTG cell disruption; 7. BL21(DE3)/pET43.1a-kN/IPTG pellets; 8. BL21(DE3)/pET43.1a-kN/IPTG supernatants; 9. Protein ladder
B: Western blot with 6xHis antibodies, 1. BL21(DE3)/pET43.1a; 2. BL21(DE3)/pET43.1a/IPTG cell disruption; 3. BL21(DE3)/pET43.1a/IPTG pellets; 4. BL21(DE3)/pET43.1a/IPTG supernatants; 5. BL21(DE3)/pET43.1a-kN; 6. BL21(DE3)/pET43.1a-kN/IPTG cell disruption; 7. BL21(DE3)/pET43.1a-kN/IPTG pellets; 8. BL21(DE3)/pET43.1a-kN/IPTG supernatants; 9. Protein ladder

NusA-6xHis-Nisin was purified and concentrated using affinity chromatography with NTA resin column(Fig. 4). Nisin collected from elution phase showed the purity of 55%. The fractions were then dialyzed and concentrated using a 10 kDa cut-off amicon. NusA-6xHis-Nisin was finally collected with 65% in purification (Table 2).

Table 2: NusA-6xHis-Nisin purification

Purification steps	Target protein content (µg)	Purity (%)	Yield (%)
Cell lysis	4436,02	20,8	100
Ni-NTA	3423,13	55,00	77,17
Amicon filtration	2950,31	65,00	66,71

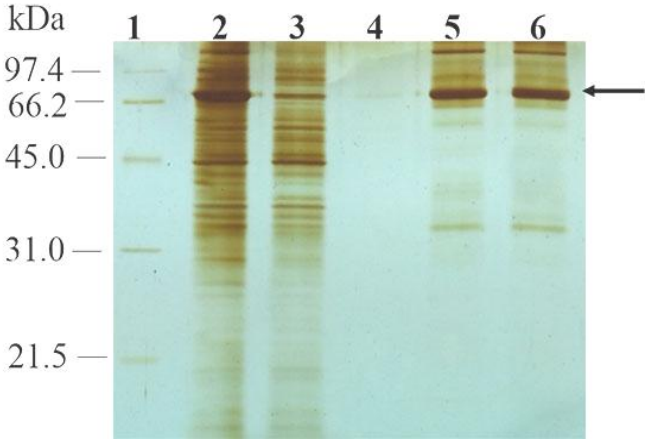

Fig. 4: NusA-6xHis-Nisin purification
1. Protein ladder; 2. BL21 (DE3)/pET43.1a-kN/IPTG cell disruption; 3. Flow phase; 4. Wash phase; 5. Elution phase; 6.Concentrated fusion nisin

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Antibacterial activity of recombinant nisin- Recombinant nisin was cleaved from NusA-6xHis-Nisin fusion protein and analyzed for anti *Listeria monocytogenes* activity. Our results showed that cleaved nisin was observed on SDS-PAGE as a band at 3.5 kDa in size (Fig. 5A) with strong antibacterial activity (Fig. 5B). Furthermore, agar diffusion assay showed that the bacterial indicator was inhibited after treatment with recombinant nisin in compare to NusA-6xHis-Nisin and ampicillin (50 µg/ml). A clear 3mm inhibition zone was observed while NusA-6xHis-Nisin did not deliver observable effect (Fig. 5C). Our result of antibacterial assay strongly indicated that recombinant nisin produced by this study inhibits the *Listeria monocytogenes*.

Consequence, recombinant nisin was diluted and tested by diffusion assay. We successfully collected nisin peptide with enterokinase treatment. The highest dilution fold was 2 (n=1) and antibacterial activity was 18.9 AU/mg.

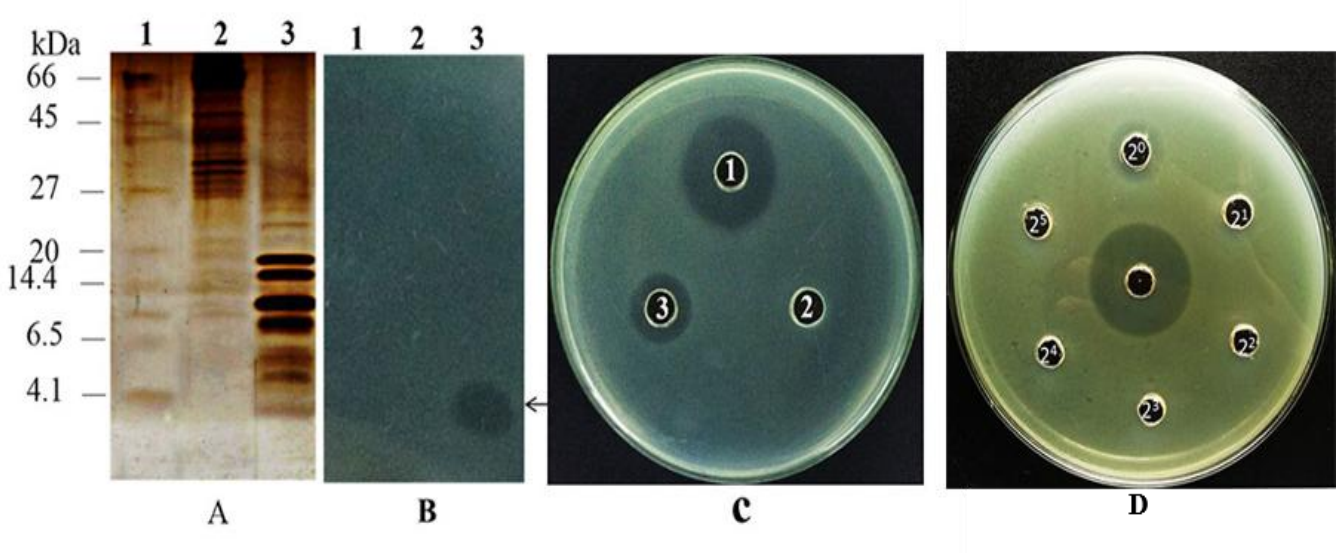

Fig. 5: Antibacterial activity of recombinant nisin.
A: Tricine SDS-PAGE fusion nisin after enterokinase treatment
B: Evaluation antibacterial activity of recombinant nisin on gel polyacrylamide
C: Evaluation antibacterial activity of recombinant nisin by agar diffusion method
1. Ampicillin (50µg/ml); 2. NusA-6xHis-Nisin; 3. Recombinant nisin
D: Specific activity of recombinant Nisin

Stability of recombinant nisin- Stability of recombinant nisin- Stability was an important aspect that makes nisin become widely applicable in many areas [12,32]. Stability of our recombinant nisin was evaluated with different pH and temperature conditions. Nisin were incubated at 30, 50, 60, 70, 80, 90, 100, and 121°C then subjected to antibacterial assay. The result showed that nisin bioactivity was stable at temperature from 30 to 90°C. Nisin bioactivity significantly decreased at higher temperatures and become inactivated at 121°C (Fig. 6A). Recombinant nisin was also treated in different pH conditions (pH1-12) for 1 hour and analyzed for antibacterial activity. The experimental results demonstrated that recombinant nisin is stable in pH range of 1-12 (Fig. 6B).

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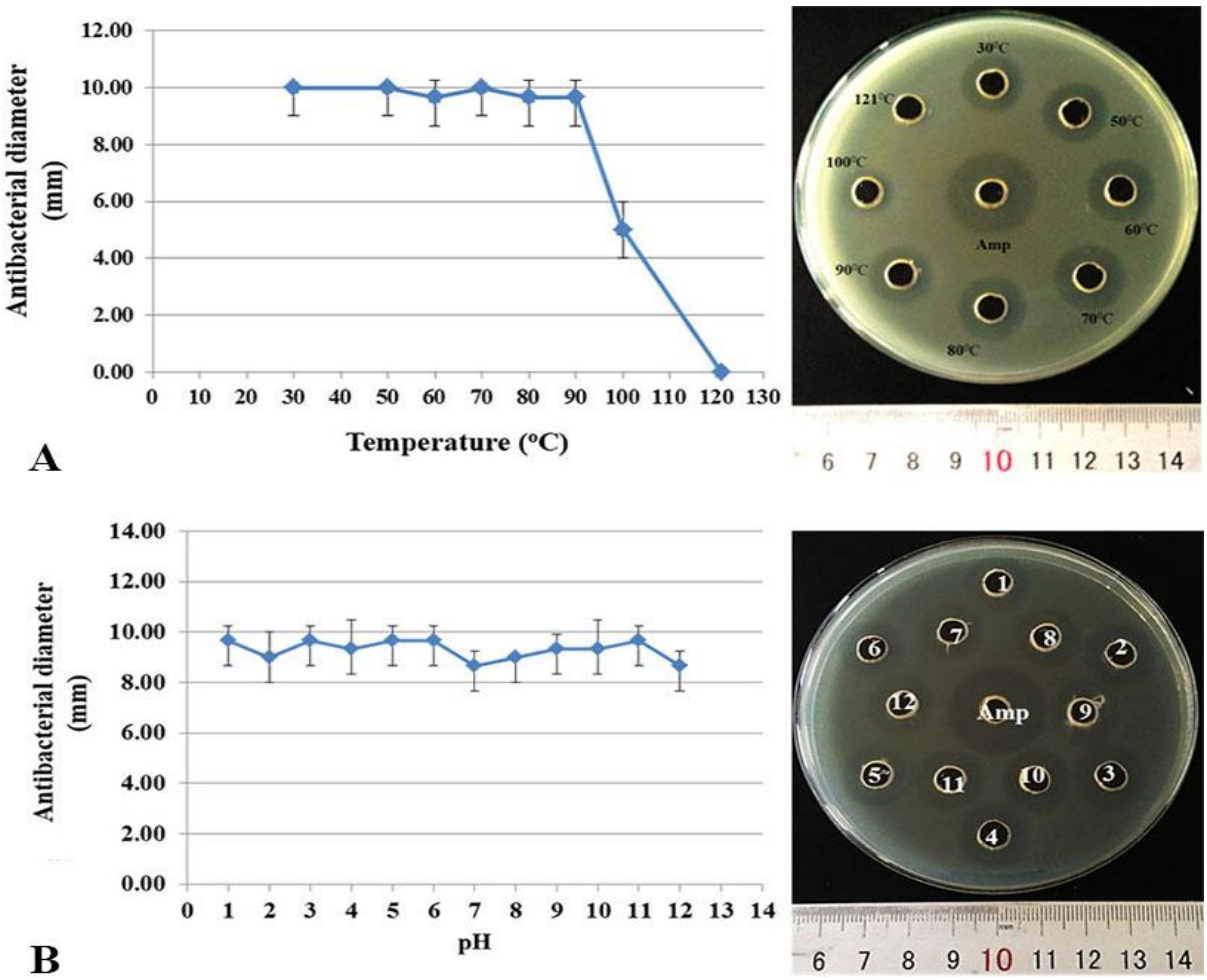

Fig. 6: Heat and pH stability of recombinant nisin
A: Heat stability of recombinant nisin
B: pH stability of recombinant nisin

DISCUSSION

Basic nisin, an antibacterial polypeptide, was originally identified from *Lactococcus lactis* [4-7]. Owning a demand for large amounts of nisin in different applications, high level expression and purification of the bacteriocin are interested to attract many scientists. Their studies focused on two main directions: improvement and optimization the culture conditions for nisin *Lactococcus lactic* strains culture and fermentation or produce nisin as recombinant protein [23,33-42]. For producing a recombinant nisin, some hosts such as *E. coli*, *Saccharomyces cerevisiae*, *Lactococcus lactis* MG1363 have been used [23,33, 43]. In this study, BL21 *E. coli* strain was used for the production recombinant nisin. *E. coli* expression system owns some advantages in producing heterologous recombinant protein such as cost effectiveness, time saving, easy culture, fast growth, and easy recovery of the recombinant protein [30,31,44]. Previous studies showed the result of expressing nisin in *E. coli* but its bioactivity [23,45].

In this study, a new strategy to produce active recombinant nisin using *E. coli* expression system was introduced. This expression method was succeeded to archive recombinant nisin protein purified with nickel affinity chromatography. Our data was also demonstrated that the stable of recombinant nisin at temperature range of 30-90^{o}C and in pH range of 1-12.

CONCLUSIONS

In summary, the study indicates that nisin protein could be expressed functionally in *E. coli* by fusing it with NusA. The productivity of NusA-6xHis-Nisin was achieved approximately 59 mg/L of induced culture (related to recombinant nisin with antibacterial activity 18.9 AU/mg).

This result demonstrated an effective production of biologically active nisin. It opened a prospect of production of nisin as recombinant protein.

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CONTRIBUTION OF AUTHORS

This study was designed by Dang Thi Phuong Thao and Nguyen Hieu Nghia. Nguyen Van Hau and Huynh Thi Xuan Mai equally contributed on data collection. Data analysis and interpretation for the work were carried out by Nguyen Hieu Nghia, Huynh Thi Xuan Mai and Nguyen Van Hau. Dang Thi Phuong Thao draftted the article and Huynh Thi Xuan Mai wrote it. The article was critically revised and approved to be published by Dang Thi Phuong Thao.

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