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Expression of the Recombinant Enterocin E-760 in *Pichia pastoris* X33 and Its Antimicrobial Activity towards *Listeria monocytogenes*

(Pengekspresan Rekombinan Enterocin E-760 pada *Pichia pastoris* X33 dan Aktiviti Antimikrob terhadap *Listeria monocytogenes*)

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ABSTRACT

Bioactive compounds such as bacteriocins have become potent and promising alternatives to chemical food preservatives for extending food shelf-life and eliminating food loss from microbial spoilage. Enterocin E-760 is a specific bacterocin belonging to class II that possesses broad spectrum antibacterial activity against both Gram-negative and Gram-positive bacteria. In this study, the enterocin E-760 gene was fused to a His-tag and cloned into the expression vector, pPICzαA, and transformed into *Escherichia coli* DH5α cells. The recombinant plasmid was isolated, linearised and transformed into competent *Pichia pastoris* X33 cells using electroporation. The *Pichia* transformants were determined using PCR and expressed under methanol induction with the highest antibacterial activity of culture supernatants reaching 40 AU/mL. Enterocin E-760 exhibited a molecular weight of approximately 5.5 kDa and was detected directly on a Tricine SDS-PAGE gel containing *Listeria monocytogenes* ATCC 35152 after ethanol precipitation at a concentration ranging from 30% to 70%. This study represented the initial stages of research into using enterocin as a biopreservative in food processing.

Keywords: Antibacterial peptide; bacteriocin; enterocin E-760; food preservation

ABSTRAK

Sebatian bioaktif seperti bakteriosin telah menjadi alternatif yang kuat dan berpotensi menggantikan pengawet makanan kimia untuk memanjangkan jangka hayat makanan dan menghapuskan kerosakan makanan daripada tindakan mikrob. Enterosin E-760 ialah bakteriosin khusus yang tergolong dalam kelas II yang mempunyai aktiviti antibakteria dengan spektrum yang luas terhadap kedua-dua bakteria Gram-negatif dan Gram-positif. Dalam kajian ini, gen enterosin E-760 telah digabungkan dengan penanda His lalu diklonkan ke dalam vektor pengekspresan, pPICzαA dan ditransformasi ke dalam sel *Escherichia coli* DH5α. Plasmid rekombinan telah dipencil, dilinear dan ditransformasikan ke dalam sel kompeten *Pichia pastoris* X33 melalui elektroporasi. Transforman *Pichia* ditentukan menggunakan PCR dan diekspreskan melalui induksi menggunakan metanol menghasilkan aktiviti antibakteria bagi supernatan kultur yang tertinggi mencapai 40 AU/mL. Enterosin E-760 mempamerkan berat molekul kira-kira 5.5 kDa dan dikesan secara langsung pada gel SDS-PAGE Trisin yang mengandungi *Listeria monocytogenes* ATCC 35152 selepas pemendakan etanol pada kepekatan antara 30% hingga 70%. Kajian ini merupakan penyelidikan pada peringkat awal menggunakan enterosin sebagai biopengawet dalam pemprosesan makanan.

Kata kunci: Bakteriosin; enterosin E-760; pengawetan makanan; peptida antibakteria

INTRODUCTION

Food spoilage can be described as any perceived changes in sensory characteristics that render food unsafe and unacceptable for consumption (Odeyemi et al. 2020). This process may be caused by many factors, but microorganisms are the primary factors that are recognised by the appearance, textural changes or off-odors and off-flavours of food (Gram et al. 2002; Odeyemi et al. 2020).

Microbial contamination of food not only significantly affects society and the economy through food loss but may also cause foodborne illnesses, many of which are still not under control and can lead to severe outbreaks. In some developing countries, like Vietnam, foodborne illnesses are promoted by conditions such as limited hygiene and safety regulations of food facilities, the production and consumption of raw or uncooked food, the use of materials that do not meet food safety standards, and the suitability of the tropical climate for microbial growth. In 2011, there were 141 foodborne outbreaks that occured in Vietnam, resulting in 38,915 cases, with 3,663 hospitalisations and 27 deaths (Carrique-Mas & Bryant 2013). Between 2009 and 2013, approximately 261 foodborne outbreaks occurred throughout 19 provinces in Southern Vietnam. Among some of the foodborne pathogens that were suspected or confirmed, bacteria were associated with the most outbreaks (over 40%), including the common species such as *Staphylococcus aureus*, *Salmonella* spp., *Shigella,* and *Escherichia coli* (Thuan et al. 2017). Hence, it is essential to eliminate the effects of microorganisms on foods to extend the shelf-life, ensure a safe, nutritional and sustainable food supply in order to safeguard human health.

One of the trends that has emerged in the worldwide food industry in recent years is the use of biopreservation method (Reis et al. 2012). Natural preservatives, such as bacteriocins, are gaining significant interest as food preservatives to replace chemical additives and maintain the food quality (Bali et al. 2016; Silva et al. 2018). Bacteriocins, which were first described by Gratia in 1925, are antimicrobial peptides that can be easily degraded by proteases of the mammalian digestive tract (Reis et al. 2012; Silva et al. 2018). To date, the benefits and prospects of using several bacteriocins have been explored and their antibacterial activity has been investigated in different food samples such as meat (Dortu et al. 2008; Lv et al. 2018), dairy food (Aguayo et al. 2016), vegetables and fruits (Lucas et al. 2006). Nisin, a member of the bacteriocin family, has been commercialised as a food additive (code E234) by the Joint Food and Agriculture Organization/World Health Organization (FAO/WHO) since 1969 (Shin et al. 2016).

Among the known bacteriocins, enterocins show interesting potential for use in food preservation and have all the notable characteristics of bacteriocins namely that they are safe (GRAS), thermal-resistant, natural and easily degraded by proteases. Enterocins also have broad spectrum antimicrobial properties including activity against many foodborne pathogenic and spoilage bacteria. Many studies have been carried out on the potential use of enterocins in preserving foods such as vegetables (Grande et al. 2005), meat (Ananou et al. 2005) and dairy products (Grande et al. 2006). Because of their significant benefits, several enterocins have been expressed as recombinant proteins to generate sufficiently large amounts for food processing (Borrero et al. 2012; Gutiérrez et al. 2006; Mesa-Pereira et al. 2018). A specific example is enterocin E-760, which demonstrates potent activity against Gram-negative and Gram-positive bacteria including some foodborne pathogens, including *Salmonella enterica*, *E. coli* O157:H7, *S. aureus,* and *Listeria monocytogenes*. Enterocin E-760 is a 5,362-Da peptide with 62 amino acid residues and an approximate pI of 8.7 (Line et al. 2008). This antibacterial peptide was first discovered from strain NRRL B-30745, which belonged to *Enterococcus durans*, *Enterococcus faecium,* and *Enterococcus hirae* group of species. However, production of this natural peptide can be time-consuming and costly, can lead to unstable activity, and may be hazardous, as some original strains release toxins. Therefore, the expression of enterocins using safe expression systems, such as *Saccharomyces cerevisiae* and *Pichia pastoris,* is an effective solution, and these systems are able to produce large amounts of recombinant protein. Enterocin E-760 has been expressed in *P. pastoris* X33 and *Chlamydomonas reinhardtii* (Arbulu et al. 2015; Quezada-Rivera et al. 2019). As the first step towards large-scale production of this bacteriocin and its application in food preservation, this study aimed to express enterocin E-760 in *P. pastoris* X33 and evaluate its antibacterial activity.

Materials and Methods

Bacterial Strains and Chemicals

The enterocin E-760 encoding gene was designed with an added polyhistidine (His-tag) towards the 3′ end and cloned into the expression vector pPICzαA (pPICzαA-EH, Genscript Corp., USA). *E. coli* DH5α (*end A1 rec A1 hsd R17 sup E44 gyp A96 thi-1 rel A1* D*lac U169* [f*80 lac ZM 15*]) was purchased from Invitrogen (UK) and used for cloning and plasmid propagation. *P. pastoris* X33 was purchased from Invitrogen and used as the recombinant protein expression system. *L. monocytogenes* ATCC 35152 was provided by the Department of Genetic Engineering (Institute of Biotechnology, VAST) and used as an indicator strain to determine antibacterial activity. Taq DNA polymerase and chemical reagents for PCR were purchased from iNtRON Biotechnology (Korea). Primers were synthesised by Invitrogen. Other chemicals were from Merck (Germany) and Difco (USA).

DESIGN OF THE SYNTHETIC ENTEROCIN E-760 GENE AND PLASMID CONSTRUCTION

The nucleotide sequence of enterocin E-760 matching the codon usage of *P. pastoris* X-33 (Arbulu et al. 2015) was used as template to design a synthetic gene. This sequence was fused to a His-tag at the 3' end, the *Eco*R I and *Xba* I restriction sites and a stop codon. Plasmid pPICZαA was chosen as an expression vector for the enterocin E-760 gene cloning upon digestion using the same restriction enzymes and ligated together to generate the plasmid pPICzαA-EH. The synthesis of the expression vector was carried out by Genscript Corp (USA).

CLONING OF ENTEROCIN E-760 GENE IN *E. coli* DH5A

After constructing and synthesising the pPICzαA-EH plasmid, it was transformed into competent *E. coli* DH5α cells using the heat-shock method (Ausubel et al. 1994) and plated onto low-salt LB medium with 25 µg/mL Zeocin for cloning and vector propagation. The plasmid was isolated and purified using a DNA purification kit (Qiagen) before being digested by *Eco*R I and *Bam*H I to confirm the presence of the designed gene in the plasmid.

TRANSFORMATION OF ENTEROCIN E-760 GENE IN *P. pastoris* X33

Competent *P. pastoris* X33 cells were prepared using a standard protocol as described by the manufacturer (Invitrogen 2010). The pPICzαA-EH plasmid was linearised using *Sac*I and transformed into competent *P. pastoris* X33 cells using electroporation under the conditions of 1.5 kV, 400 Ω, and 25 µF. *P. pastoris* X-33 cells containing the expression vector were screened in YPDS agar supplemented with 100 µg/mL Zeocin and incubated for 3 to 4 days at 30 °C. Subsequently, total DNA from the recombinant strain was isolated and used as the template for PCR using AOX1 primers (Forward primer 5′-GACTGGTTCCAATTGACAAGC-3′; Reverse primer: 5′ GCAAATGGCATTCTGACATCC-3′) to analyse whether the gene of interest had integrated into the *Pichia* genome. The PCR reactions were carried out using the program which consisted of heating at 94 °C for 2 min, then 25 cycles of denaturation at 94 °C for 1 min, annealing at 55 °C for 1 min, and extension at 72 °C for 1.5 min followed by a final extension at 72 °C for 10 min. The PCR products were confirmed by running on a 0.8% agarose gel. Recombinant *P. pastoris* strains containing enterocin E-760 gene were chosen for induction and expression.

EXPRESSION OF HIS-TAGGED ENTEROCIN E-760 IN *P. pastoris* X33

In order to express the His-tagged enterocin E-760, we used a previously described method (Invitrogen 2010). Briefly, the recombinant *P. pastoris* X33 strains containing the enterocin E-760 gene were activated in BMGY medium and grown at 28 to 30 °C in a rotary shaker (200 rpm) until the cell density at 600 nm reached 2 to 6 (log-phase growth). The cells were harvested by centrifuging at 4500 g for 5 min at room temperature and resuspended in BMMY medium to an OD_{600} of 1.0 for induction. Methanol (100%) was added to a final concentration of 0.5% every 24 h to maintain induction. After 48 h of induction, the expression culture was harvested to analyse the antibacterial activity and to detect the recombinant protein band on a Tricine SDS-PAGE gel.

AGAR DIFFUSION ASSAY TO DETERMINE ANTIBACTERIAL ACTIVITY

A cell suspension of *L. monocytogenes* ATCC 35152 was incubated in BHI medium at a final concentration of 10^5 CFU/mL. After homogenising, the agar was poured into petri dishes and cooled to 4 °C for 30 min, before holes of 6 to 8 mm in diameter were punched in the agar using a pipette tip to contain 50 to 200 µL of cell-free supernatant from *P. pastoris* X33 culture. After diffusion at 4 °C for 2 h, the plates were incubated at 37 °C overnight. The antimicrobial activity was determined by measuring the diameter of the inhibition zone around the hole and expressed as arbitrary units (AU) per mL according to a previous method (Todorov et al. 2007).

$$\text{Antibacterial activity (AU/ML)} = 2^N/V$$

where 2 is the dilution factor; n is the last dilution that resulted in an inhibition zone of ≥ 2 mm in diameter; V is the volume of supernatant.

PURIFICATION OF PROTEIN BY ETHANOL PRECIPITATION

Ethanol was added drop by drop to five flasks containing the equivalent volume of recombinant supernatant to final concentrations of 30, 40, 50, 70, and 80%. The mixtures were stirred for 30 min and centrifuged at 9000 g for 10 min. The concentrated protein was dried in a SpeedVac concentrator for 1 h to remove residual ethanol before being resuspended in phosphate buffer (pH 5) using 1/10 of the original culture volume, and the antibacterial activity was determined.

TRICINE SDS-PAGE

The recombinant peptide was analysed on a Tricine SDS-PAGE gel, according to a previous method (Schägger 2006). To directly determine the antibacterial activity of the protein bands, the gel was placed in BHI agar and covered with 10 mL BHI medium inoculated with 10^6 CFU/mL of *L. monocytogenes* ATCC 35152 culture. The plates were incubated at 37 °C overnight, and the inhibition zones were observed.

RESULTS AND DISCUSSION

DESIGN OF SYNTHETIC GENE ENCODING ENTEROCIN E-760 AND PLASMID CONSTRUCTION

Most bacteriocins are peptides smaller than 10 kDa (Meade et al. 2020), therefore, they can be easily degraded by proteases in expression hosts. Some fusion tags such as His-tag, cellulose-binding domain (CBD), maltose-binding protein, thioredoxin or the small ubiquitin-related modifier can be conjugated to bacteriocins to eliminate proteolytic activity. These fusion partners also play important roles in maintaining protein stability, producing a target protein with the correct biological structure, and helping with protein detection and purification (Mesa-Pereira et al. 2018). In this study, we designed a recombinant gene fused to a His-tag encoding nucleotide sequence towards the 3′ end. By using NEBcutter V2.0 software, the restriction sites *Eco*R I and *Xba* I were chosen and added to the 5′ end and 3′ ends, respectively. A stop codon was inserted between the His-tag sequence and the *Xba* I restriction site to generate a 219-bp His-tagged enterocin E-760 gene (Figure 1(a)). The designed gene (indicated in yellow in Figure 1(b)) was ligated to the pPICZαA plasmid, which contains the methanol-inducible alcohol oxidase promoter (PAOX1), allowing gene expression. Some bacteriocins have also been designed with a His-tag for expression in *E. coli* and *P. pastoris* (Li et al. 2020; Olejnik-Schmidt et al. 2014). In 2019, a recombinant gene coding enterocin E-760 was designed with an added His-tag and an in-frame thrombin cutting site and expressed in *Chlamydomonas reinhardtii* (Quezada-Rivera et al. 2019).

A

gaattcAacagatggtactgtaactccgctgctggtggtgttggtggtgctgctgtttgtggtttggctggttat
EcoRI **Enterocin E-760**

Gttggtgaggctaaagaaaacattgctggtgaggttagaaagggttggggtatggctggtggtttcactcataa
Enterocin E-760

caaggcttgtaagtccttcccaggttctggttgggcttctggt**catcatcatcatcatcat** **tga** **tctaga**
6x His **XbaI**

B

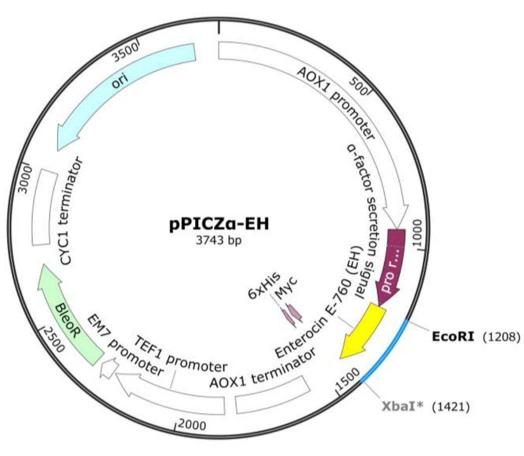


FIGURE 1. The design of enterocin E-760 (*ent*) gene (a) and plasmid pPICzαA-EH containing *ent* gene (b) for expression in *P. pastoris* X33

CLONING THE ENTEROCIN E-760 GENE IN *E. coli* DH5A

The expression vector was transformed into *E. coli* DH5α to generate multiple copies of the plasmid for expression in *P. pastoris*. After isolation and purification, the plasmid was digested by two restriction enzymes and analysed on a 0.8% agarose gel to check the presence of the designed gene. Because *Xba* I was blocked by *dam* methylation in *E. coli,* we used *Bam*H I instead (Nelson et al. 1993). It could be observed from the results that pPICzαA-EH plasmid was cut into two DNA fragments, the smaller one of which was about 600 bp and included enterocin E-760 gene (219 bp) and a part of pPICzαA that was cut by *Bam*H I (406 bp) (lane 1, Figure 2). Therefore, the pPICzαA-EH plasmid containing the enterocin E-760 gene was successfully cloned in *E. coli* and propagated for transformation in *P. pastoris* X33.

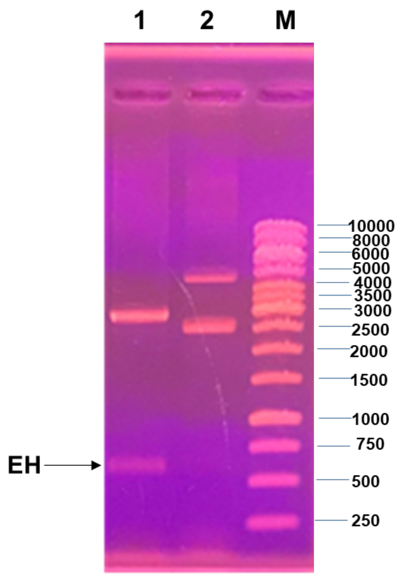


FIGURE 2. Analysis of *ent* gene in plasmid pPICzαA-EH digested by *Eco*R I and *Bam*H I. Lane 1, pPICzαA-EH digested by *Eco*R I and *Bam*H I; Lane 2, pPICzαA-EH isolated from *E. coli* DH5α; and M, 1kb DNA ladder

TRANSFORMATION AND EXPRESSION IN *P. pastoris* X33

P. pastoris X33 was chosen as the host cell for expression because it is safe, easy to cultivate and can produce a large amount of correctly folded and biologically active protein (Pedro et al. 2015). To secrete the target protein, pPICZαA plasmid was used as an expression vector, as it contains a Zeocin-resistance gene for selection. First, pPICzαA-EH was linearised by *Sac* I for the integration of the plasmid into the *P. pastoris* genome. After transformation, the cells were screened on YPDS agar supplemented with 100 µg/mL of Zeocin. The results illustrated that only transformants containing pPICzαA-EH had the ability to grow on Zeocin plates. The transformation of the *P. pastoris* X33 genome with a linear foreign plasmid can create Mut^+ (methanol utilization plus) or Mut^S (methanol utilization slow) phenotype that relates to the induction by methanol. Mut^+ strains with the AOX gene can use methanol in the range from 0.5% to 2.5% for both biomass growth and protein production, but they are sensitive to temporary high methanol concentrations (Karbalaei et al. 2020; Krainer et al. 2012). Meanwhile, Mut^S strains grow slowly in medium containing methanol and need a long time for induction; however, they are less sensitive and more stable in low methanol concentration (Krainer et al. 2012). Therefore, it is necessary to determine the Mut phenotype and design a strategy to improve the protein expression of each phenotype, which can be detected by PCR using the AOX primers. If the transformant is a Mut^+ integrant, two amplification bands can be seen on agarose gel where one is the gene of interest and the other is the AOX1 gene (approximately 2.2 kb). However, with the Mut^S phenotype, only one band is detected because the AOX1 gene is lost (Invitrogen 2010). The results of the PCR analysis showed that all *Pichia* integrants had the Mut^+ phenotype (Figure 3) where one band contained the gene of interest (219 bp) and the AOX1 gene sequence in pPICzαA (500 bp), and the other was the AOX1 gene (2.2 kb). The Mut^+ strains were then expressed using methanol induction for enterocin E-760 production.

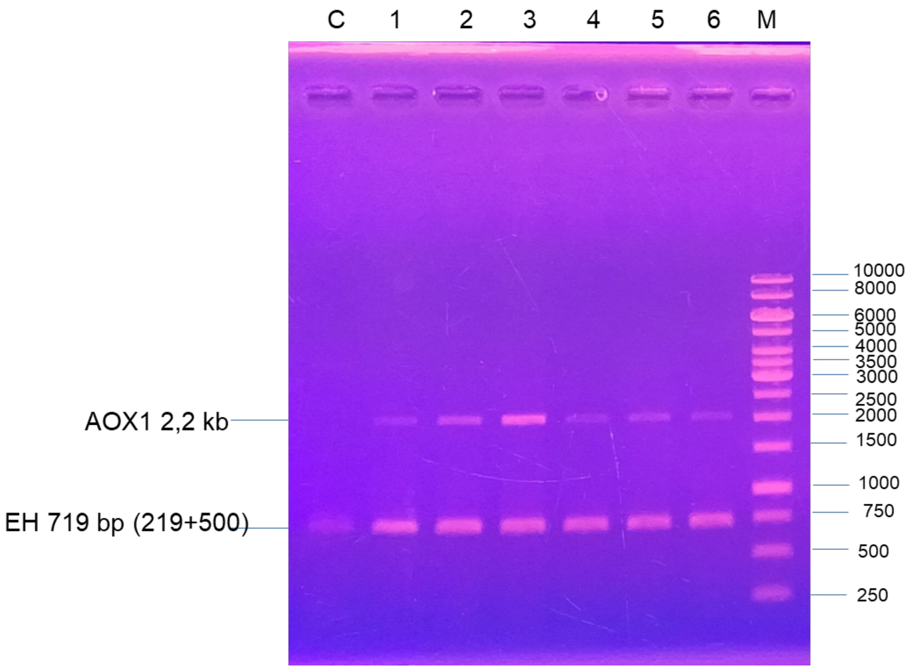


FIGURE 3. PCR analysis of the *ent* gene in *Pichia* genome. Lane M, 1kb DNA ladder; lane C, PCR product of the amplification using plasmid pPICzαA-EH as template; Lanes 1 to 6, DNA from *Pichia* transformants in lanes 1-5 as templates

In theory, the expected molecular weight of the His-tagged enterocin E-760 is approximately 5.5 kDa. To confirm the appearance of the antibacterial peptide, *Pichia* culture supernatants harvested after 48 h of induction were analysed on a Tricine SDS-PAGE gel. The results in Figure 4 demonstrated that a 5.5 kDa-protein band (expected enterocin E-760 band) was observed in most lanes of 10 strains (except E2, E5 and E7). This band was also not detected in the expression culture of the control strain P41. In addition, the anti-*listerial* activity of the supernatants from these strains was determined by agar diffusion assay (Figure 5). All the strains containing the 5.5 kDa-protein showed antibacterial activity at different levels, and the highest activity of 40 AU/mL was detected in the E1 strain. To confirm the presence of enterocin E-760, proteins in the culture supernatant of the E1 strain were ethanol precipitated and the antibacterial activity was directly analysed on Tricine polyacrylamide gel.

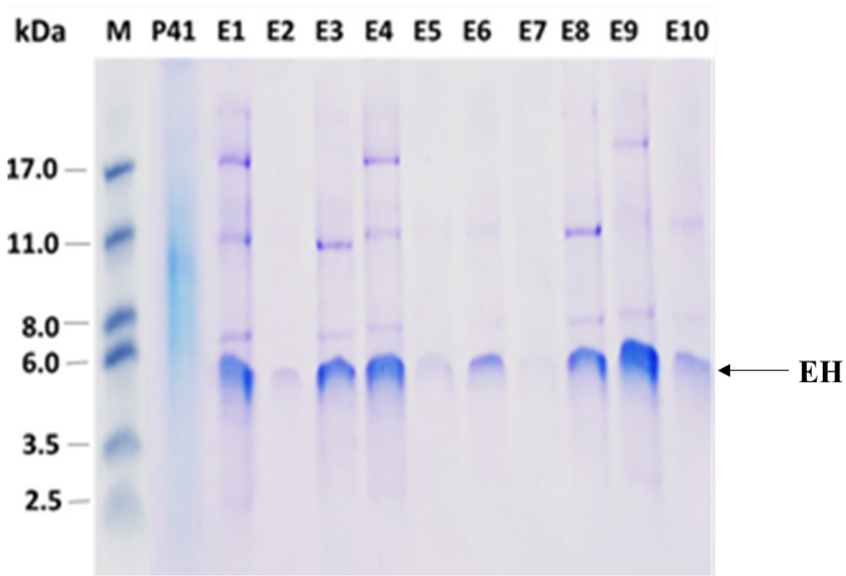


FIGURE 4. Tricine SDS-PAGE analysis of culture supernatants from recombinant *P. pastoris* X33. Lane M, protein molecular weight marker; P41, *P. pastoris* X33 containing pPICzαA without *ent* gene; Lanes E1 to E10, *P. pastoris* X33 strains containing pPICzα-EH

A B

FIGURE 5. Analysis of antibacterial activity of supernatants of recombinant strains for E1 to E5 strains (A) and E6 to E10 strains (B). ĐC-, *P. pastoris* X33 containing pPICzαA without *ent* gene; E1 to E10, *P. pastoris* X33 strains containing pPICzα-EH

In previous study, enterocin E-760 was expressed in *P. pastoris* X33; however, the supernatant of the recombinant strain did not show antibacterial activity. In this study, the *Xho*I and *Not*I restriction sites were added to the synthetic gene in order to generate the nucleotide fragment containing the Kex2 signal cleavage fused to the mature synthetic enterocin E-760 gene. The Kex2 secretion signal may affect the secretion of fused peptides or proteins in *Pichia*. Besides, the Kex2 P1' site residue can affect the yields of many recombinant proteins (Arbulu et al. 2015). In addition, some methods in the previous study have not been clearly provided such as the condition of electroporation, the use of BMGY with glycerol as carbon source to activate the recombinant strains before the induction, and the determination of *Pichia* phenotype related to the suitable methanol concentration for induction. These factors can significantly affect the production and activity of the recombinant protein. In 2019, enterocin E-760 was also successfully expressed in *C. reinhardtii*, and the recombinant peptide demonstrated antibacterial activity against some Gram-negative and Gram-positive strains belonging to species such as *S. aureus*, *E. faecium*, *Pseudomonas aeruginosa,* and *Klebsiella pneumoniae* (Quezada-Rivera et al. 2019).

PURIFICATION OF PROTEIN BY ETHANOL PRECIPITATION

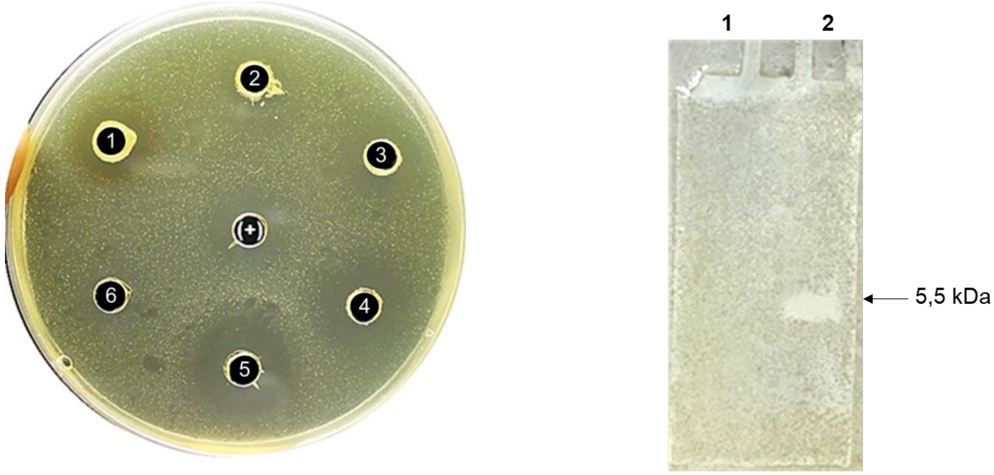
Ethanol precipitation is an effective method of concentrating protein samples during downstream processing. At each ethanol concentration, different proteins show different solubility. Therefore, in this research, we used ethanol in the 30% to 80% concentration range to determine the most suitable concentration for enterocin E-760 precipitation. The results provided in Table 1 illustrated that there was an increase in the activity of enterocin E-760 when ethanol was added gradually. Antibacterial activity reached a peak of about 320 AU/mL with 70% ethanol treatment and was maintained at stable levels with 80% ethanol. The results also showed that enterocin E-760 was completely harvested by adding ethanol to a final concentration of 70%.

TABLE 1. The antibacterial activity of protein precipitated using different final concentrations of ethanol

	Ethanol final concentration (%)					
	0	30	40	50	70	80
V (mL)	100	10	10	10	10	10
AU/mL	39,6±4,2	18,1±2,4	77,5±4,2	159,3±4,5	320,6±4,8	320±3,4

To confirm this result, we harvested precipitated protein from each fraction after adding ethanol to final concentrations of 30, 40, 50, 60, 70, and 80%, and determined the antibacterial activity. Protein from the 30 to 70% ethanol fractions showed antibacterial activity, and the largest inhibition zone was seen with the 70% fraction. Ethanol was then continuously added to a final concentration of 80%; however, the proteins precipitated from this fraction did not display antibacterial activity (Figure 6(a)). Consequently, the supernatant of the recombinant strain was precipitated by adding ethanol to final concentrations ranging from 30% to 70% to gain the maximum amount of enterocin E-760. In another study, a bacteriocin from *Lactobacillus rhamnosus* strain 68, called rhamnosin A, was precipitated from the crude supernatant using 90% ethanol and reversed-phase chromatography (Dimitrijević et al. 2009).

The protein obtained by ethanol precipitation was fractionated on a Tricine polyacrylamide gel before being assayed for activity against *L. monocytogenes* ATCC 35152. Only one protein band in lane 2 (the precipitated protein) resulted in an inhibition zone for *L. monocytogenes* ATCC 35152, and it had a molecular weight of 5.5 kDa, as expected for enterocin E-760 (Figure 6(b)). This result proved that enterocin E-760 was successfully expressed and obtained in a biologically active form. Furthermore, the stability of enterocin E-760 during solvent treatment demonstrated the considerable potential for its ability to withstand further purification steps and the application of this antibacterial peptide in food preservation.



A B

FIGURE 6. Analysis of antibacterial activity of proteins at each fraction (a) and protein in 30 to 70 % fraction after precipitation in Tricine SDS-PAGE gel coated with *L. monocytogenes* ATCC 35152 (b)

(a) 1: Crude supernatant; 2: ethanol 30 %; 3: ethanol 40 %; 4: ethanol 50 %; 5: ethanol 70 %; 6: ethanol 80 %; (+): Zeocin

(b) 1: Precipitated protein from *P. pastoris* X33 containing pPICzαA without *ent* gene

2: Precipitated protein from *P. pastoris* X33 containing pPICzαA-EH (E1)

Conclusion

In this study, we demonstrated the *P. pastoris* X33 expression of enterocin E-760, which is a promising, safe bioactive compound for food preservation. The nucleotide sequence of this antibacterial peptide was fused with a His-tag sequence and inserted into the pPICzαA plasmid for expression in *P. pastoris* X33. The highest antibacterial activity, as analyzed by agar diffusion method, reached 40 AU/mL. After precipitation with ethanol concentrations ranging from 30% to 70%, the antibacterial activity of enterocin E-760 was determined to be 320 AU/mL. The recombinant protein was estimated to be approximately 5.5 kDa and illustrated anti-*Listerial* activity in Tricine SDS-PAGE gel. Some expression conditions of the recombinant strain can be optimised for high-level production of the recombinant protein. Additionally, enterocin E-760 can be purified using different methods, such as affinity chromatography, for further studies as well as for its practical application.

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