



Antimicrobial Activity and Identification of Gene Encoding Enterocin *Enterococcus faecalis* K2B1 Isolated from Toraja's Belang Buffalo Milk

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ABSTRACT

Background: Enterocin in *Enterococcus* is coded by enterocin encoding genes namely A, B, P and L50A / B. The purpose of this study was to identify enterocin gene encoding *enterococcus faecalis* K2B1 probiotic candidate from Belang Toraja buffalo milk and antimicrobial activity to *S. typhi*. **Methods:** identification of enterocin gene encoding using ent A, B, P and L50A / B, partial purification using ammonium sulfate on 80 % concentration and antimicrobial activity against *Salmonella typhi* using disk diffusion method. The results of PCR amplification are then sequenced and BLASTx on NCBI. **Result:** Antimicrobial activity of Precipitate and crude against *S. typhi* are 193 and 201 respectively. Identification gene encoding enterocin shows that Ent A, B and P cannot be amplified and only EntL50A / B can be amplified with a sequence size of 86 bp. The sequence of enterocin encoding genes in *E. faecalis* K2B1 has 94% similarity with hypothetical protein EB34_00789 *E. faecalis* on GenBank with accession number RBR60004.1 **Conclusion:** EntL50A / B *E. faecalis* K2B1 has a size of 86 bp and is 94% identical to the hypothetical protein EB34_00789 and Enterocin can be used as antimicrobial or bio preservative.

Aktivitas Antimikroba dan Identifikasi Gen Penyandi Enterocins isolat *Enterococcus faecalis* K2B1 dari Susu Kerbau Belang Toraja

ABSTRAK

Background: Enterocins merupakan senyawa antimikroba yang dihasilkan oleh *Enterococcus* dan dapat menghambat pertumbuhan patogen. Enterocins dikode oleh gen penyandi enterocins yaitu ent A, B, P dan L50A/B. Tujuan penelitian ini adalah untuk mengetahui aktivitas antimikroba enterocins terhadap *S. Typhi* dan mengidentifikasi gen penyandi enterocins *Enterococcus faecalis* K2B1 kandidat probiotik dari susu kerbau Belang Toraja. **Metode:** metode yang digunakan pada penelitian ini yaitu *E. faecalis* K2B1 ditumbuhkan pada kondisi optimum, setelah itu dilakukan purifikasi parsial menggunakan ammonium sulfat konsentrasi 80 %, kemudian diuji aktivitasnya terhadap *S. typhi* menggunakan disk diffusion method. Identifikasi gen penyandi enterocins menggunakan ent A, B, P dan L50A/B dan dilanjutkan dengan BLASTx pada NCBI. **Hasil:** hasil penelitian menunjukkan bahwa presipitat dan crude enterocins (enterocins) mampu menghambat pertumbuhan *S.typhi* yaitu 193 dan 201 mm, aktivitas enterocins sebagai antimikroba telah hilang setelah penambahan Poteinase-K. Hasil identifikasi gen penyandi menunjukkan bahwa ent A, B dan P tidak dapat teramplifikasi dan hanya EntL50A/B yang dapat teramplifikasi dengan ukuran sekuen 86 bp. Sekuen gen penyandi enterocins pada *E. faecalis* K2B1 memiliki similaritas 94 % dengan *hypotethical protein* EB34_00789 *E. faecalis* pada GenBank dengan accession number RBR60004.1. **Kesimpulan:** Enterocins dari *E. faecalis* K2B1 mampu menghambat pertumbuhan *S. typhi* dan hasil identifikasi gen penyandi Enterocins menunjukkan bahwa EntL50A/B *E. faecalis* K2B1 memiliki ukuran 86 bp, dan 94% identik dengan *hypotethical protein* EB34_00789.



Introduction

Bactericins is an antimicrobial compound produced by Lactic Acid Bacteria (LAB) (Arqués et al., 2015; Umniyati et al., 2006). Tambekar & Bhutada (2010) found that LAB from cow, buffalo and goat milk which can inhibit *S. typhi* Rizqiati et al. (2015) found 41 LAB isolates from buffalo milk in North Sumatra and were able to inhibit *S. typhi*.

One of the member LAB can produce bacteriocins, called enterocins is Enterococcus genus (Maky et al., 2015). Enterocin is a protein from the gene encoding enterocins in Enterococcus bacteria located on the chromosome or plasmid. Genes encoding enterocins can be detected using ent A, B, P, and L50A/B primers. Several studies of genes encoding enterocins for probiotic and bio preservative candidate have been reported by Banwo et al. (2013) on *E. faecium*, Sarra et al. (2013) against *E. faecium*, Braïek et al. (2017) against *E. lactis* and Vimont et al. (2017) against *E. faecium*.

The research related to the gene encoding Enterocins against *E. faecalis* in Indonesia has not been reported. *Enterococcus faecalis* K2B1 is an isolate obtained from Indonesian Toraja's Belang Buffalo milk and has been known to have potential as a probiotic. In addition, the optimum conditions for producing and Enterocins characteristics produced by these isolates have also been known to be potential as bio preservatives (Alang et al., 2020). However, studies related antimicrobial activity against *S.typhi* by *E. faecalis* and genes encoding enterocins from these isolates have not been reported.

This aims of this study are to know antibacterial activity of enterocins against *S. typhi* and detect the gene encoding Enterocins from *Enterococcus faecalis* K2B1 using ent A, B, P, and L50A / B primers.

Methods

The research has been conducted in Microbiology Laboratory, Faculty of Mathematics and Natural Science, Universitas Brawijaya, in 2019. Tools used to carry out the research include PCR (Eppendorf), electrophoresis (Mupid-exU), UV-Geldock, centrifuge (Hettich), vortex spindown, Micropipette, incubator shaker, *de Man Rogosa Sharpe Agar* (MRSA) (Merck, Darmstadt, Germany), *de Man Rogosa Sharpe Broth* (MRSB) (Merck, Darmstadt, Germany), Ammonium sulphate, Milipoure, cellophane membrane, NA, blank disk (Oxoid), Proteinase-K, *S.typhi*, *i-genomic Soil DNA Extraction Mini Kit* (iNtRon), master mix (*Green Go Taq*, Promega), *Nucease Free Water* (NFW), *ethidium bromide* (etbr), DNA template and primer gene encoding enterocins *EntA* f (5'-GGT ACC ACT CAT AGT GGA AA-3') r (5'- CCC TGG AAT TGC TCC ACC TAA-3'), *EntB* f (5'- CAA AAT GTA AAA GAA TTA AGT ACG-3') r (5'- AGA GTA TAC ATT TGC TAA CCC/TCC TGC AAT ATT CTC TTT AGC-3'), *EntP* f (5'- GCT ACG CGT TCA TAT GGT AAT-3') r (5'- TCC TGC AAT ATT CTC TTT AGC) and *EntL50A/B* f (5- ATG GGA

GCA ATC GCA AAA TTA-3') r (5'- TTT GTT AAT TGC CCA TCC TTC / CCT ACT CCT AAG CCT ATG GTA-3').

Procedure

The research procedure consisted of partial purification, antimicrobial activity assay, DNA extraction, and gene encoding enterocin.

Partial Purification

Enterocins production was carried out on MRS broth pH eight media using a shaker incubator 37 °C, 150 rpm, 13 hours. LAB cultures were centrifuged at 4 °C, 10000 g, 20 minutes. The supernatant was filtered using millipore 0.22 µm, and the pH was neutralized to 6.5 using NaOH. The filtrate was then precipitated with ammonium sulphate 80%, 4 °C overnight using a stirrer, centrifuged 10000 g at 4 °C, and 20 minutes. The precipitates were added with a phosphate buffer of 0.2 M pH of 6.7 with a ratio of 1: 1. then dialysis using a 5 kDa MWCO membrane celofan at 4 °C overnight using a stirrer.

Antibacterial Activity Against *S. typhi*

The precipitate and crude enterocin analysis was then assayed for antimicrobial activity against *S. typhi* using the *disk diffusion method*. The surface of Petri dishes containing soft NA (0.8%) was spread 100 µL with *S.typhi* density of 10⁶ cells/mL. The precipitates or crude enterocin was dropped 50 µL on a blank disk (Oxoid), then dried until all was absorbed by the disc, incubated at 4 °C, 30 minutes. The cultures were incubated at 37 °C, 24 hours. The antimicrobial activity is then calculated (Avaiyarasi et al., 2016; Thirumurugan et al., 2015).

$$AU = Lz - Ls/V$$

Lz = clear zone area (mm)

Ls = well area (mm)

V = Sample volume (mL)

Determination of crude enterocin as a protein was characterized by Proteinase-K's addition to the crude enterocin, then antimicrobial activity against *S. typhi* was assaying.

DNA Extraction

The genetic material of chromosomal DNA isolates *E. faecalis* K2B1 was extracted using *i-genomic Soil DNA Extraction Mini Kit* with slight modification. Cultures in MRSA media with a 24-hour slant tube were taken for five ose and put in a 1.5 mL volume micro tube filled with 50 µL of sterile distilled water. The suspension was pipetted and transferred to a grinder tube, added 400 µL of EG buffer, and then vortexed at maximum speed for 20 minutes.

The sample suspension was centrifuged at 13,000 rpm for one minute. The apparent undercoat was removed and transferred to a new, sterile, 1.5 mL micro-tube. The

suspension was added with EPT buffer as much as 100 µL and vortexed for five minutes, incubated on ice for 10 minutes, then centrifuged at 13,000 rpm for three minutes.

The supernatant was put into a sterile micro-tube, added with 300 µL of EB buffer, inverted six times. The suspension was added with 300 µL of absolute ethanol then inverted 5-6 times and spindown. The suspension was taken 800 µL and put into a spin column, centrifuged at 13,000 rpm for one minute. The filtrate was removed and centrifuged at 13,000 rpm for 1 minute. Collection tube removed and replaced with a new one.

The 700 µL EWA buffer was added to the collection tube and then centrifuged at 13,000 rpm for one minute. The 700 µL EWB buffer was added and then centrifuged at 13,000 rpm for one minute. The filtrate is removed and then centrifuged at 13,000 rpm for one minute. This is so that DNA is completely clean from ethanol.

The spin column was transferred to a new microtube, added 50 µL of EE buffer, incubated at room temperature for one minute, and the microtube's lid was left open to evaporate the remaining ethanol. The microtube was closed and centrifuged at 13,000 rpm for one minute, and the colloum spin was discarded.

Detection of Genes Encoding Enterocins

Gene encoding enterocin was amplified by PCR using specific primers (Batdorj et al., 2006; Foulquie-Moreno et al., 2003). Each PCR reaction consisted of 13.0 µL of PCR master mix (green go Taq, Promega), 1.0 µL primer (10 pmol), 5.0 µL NFW and 5.0 µL DNA template (50 ng). A 35-cycle PCR program was used, consisting of the initial denaturation, denaturation, annealing, extension and final extension as in Table 1. The amplicons were electrophoretic on 2% agarose using 100 bp marker and running 90 Volt for 1 hour. The amplicon was subsequently sequenced by First BASE Laboratories, Malaysia.

Table 1. Specific primary sequences and PCR Amplification of Genes Encoding Enterocins

Genes	Amplification (35 cycles)
<i>EntA</i>	Initial denaturation 95 °C, 5 minute; Denaturaton :95 °C, 30 second; <i>Annealing</i> : 50 - 62 °C, 30 second, Extension: 72 °C, 30 second
<i>EntB</i>	Initial denaturation 95 °C, 5 minute; Denaturaton:95 °C, 30 second; <i>Annealing</i> : 50 - 62 °C, 30 second, Extension: 72 °C, 30 second
<i>EntP</i>	Initial denaturation 95 °C, 5 minute; Denaturaton: 95°C, 30 second; <i>Annealing</i> : 50 - 62 °C, 30 second, Extension: 72 °C, 30 second.
<i>Ent</i> <i>EntL50A/B</i>	Initial denaturation 95 °C, 5 minute; Denaturaton: 95°C, 30 second; <i>Annealing</i> : 48 °C, 30 second; Extension: 72 °C, 30 second.

Data Analysis

The AU was calculated for antimicrobial activity test results, while the coding gene sequencing was carried out by BLASTX on NCBI (National Center for Biotechnology Information) and compared with nucleotide sequences in the NCBI GenBank database.

Results

The antimicrobial activity of precipitates and crude enterocins showed that crude enterocins was more effective at inhibiting *S. typhi* than precipitates (Figures 1 and Figures 2).

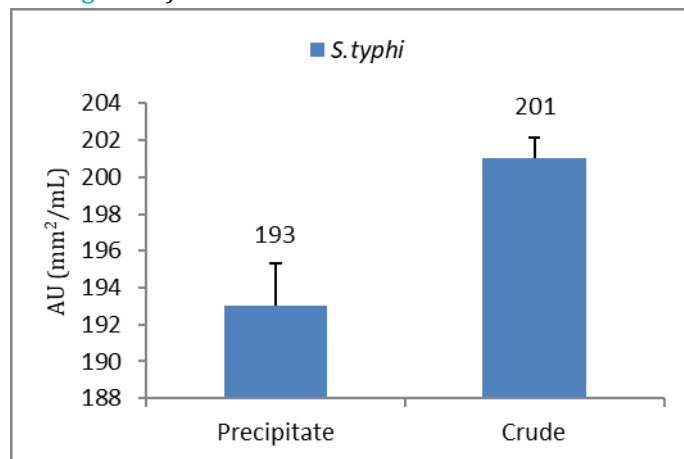


Figure 1. Arbitrary Unit Precipitates and Enterocins against *S. typhi*

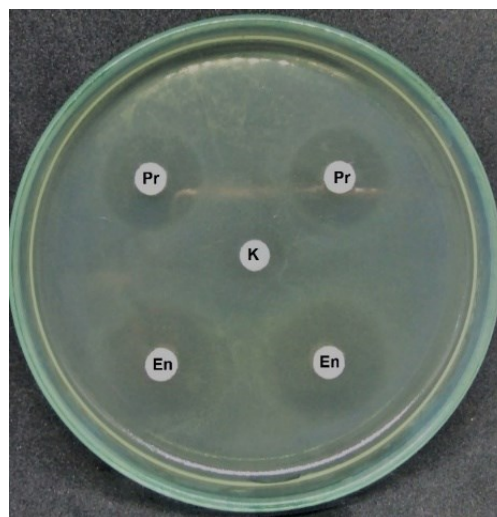


Figure 2. Arbitrary Unit of Precipitates and crude enterocin against *S. typhi* in Petri dishes (P: Precipitates, En: Enterocins, K: Antibiotic or control).

Enterocins activity after Proteinase-K addition showed that the enterocins no had antimicrobial activity against *S. typhi* (Figure 3). Identification of a gene encoding Enterocins *E. faecalis* K2B1 showed that only one gene could be detected, namely ntl50A / B. In contrast, the other encoding gene could not be amplified (Figure 4).

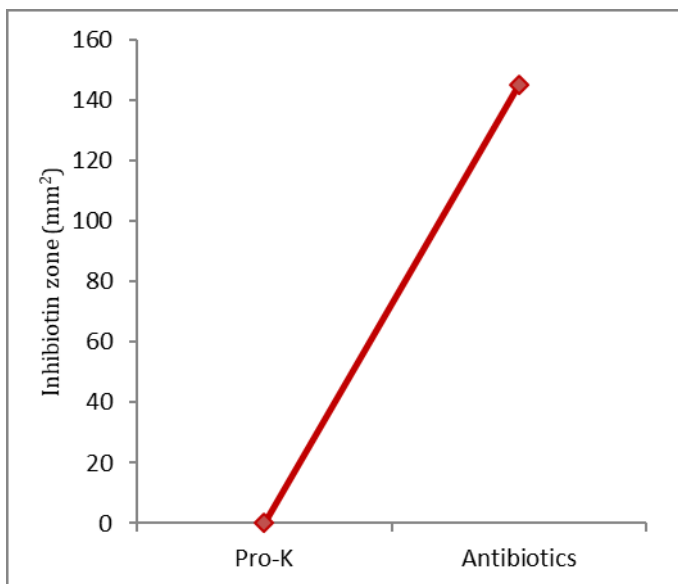


Figure 3. Characteristics of crude Enterocins (enterocin) after the addition of Proteinase K

The Ent L50A/B sequencing and amplification results showed that the gene's nucleotide sequence was concise, around 86 bp (Figure 5). The next sequenced nucleotide sequences were in BALSTX on NCBI. The blast results indicated that the classified EntL50A / B gene was similar to the *Hypothetical Protein* in the gene bank (Figure 6).

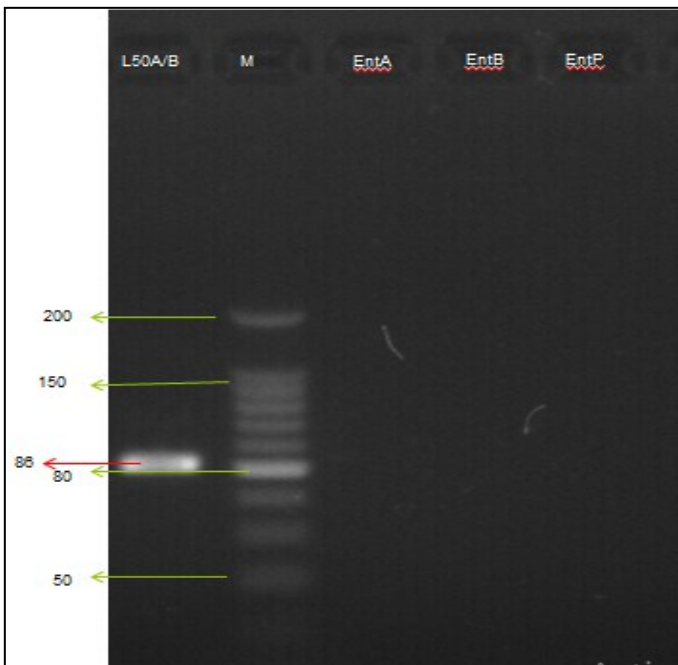


Figure 4. The PCR amplification band of gene encoding enterocin

Discussion

Enterocins production is carried out according to its optimum conditions. *Partial purification* was carried out by adding *ammonium sulphate* to produce precipitates, followed by dialysis using a cellophane membrane with 5 kDa *molecular weight cut off membrane pore* (MWCO). In

cold conditions, this process is carried out in other compounds that are thought a protein, do not denaturation due to hot temperatures. The purpose of dialysis is to remove the influence of salt from the precipitation process. The antimicrobial activity of precipitates and enterocins was tested against *S. typhi*. The optimum production of bacteriocin is indicated by the amount of zone of inhibition as antibacterial, expressed in *arbitrary units* or AU (mm² / mL).

The results show that AU of precipitates and Enterocins are 193 mm²/mL and 201 mm²/mL, respectively (Figures 1 and 2). This was similar to Arqués et al. (2015), which states that bacteriocins are used to control pathogenic microorganisms. The bacteriocin produced by LAB can be bactericidal or bacteriostatic, incredibly close related bacteria. Enterocins is a cationic molecule, positively charged, while the cell membrane bacterial is negatively charged. This facilitates the Enterocins to bind in the cell membrane of bacterial, then makes pores in the membrane cause the cell to die. The enteriocin produced by *E. faecalis* K2B1 was antibacterial activity assay after adding proteinase-K. The results showed that no inhibition zone was formed. This means that the enterocins no has antibacterial activity against *S. typhi* (Figure 3).

The same research was also conducted by Ferreira et al. (2007), Dezwaan et al. (2007), Banwo et al. (2013), and Udhayashree et al. (2012) namely by adding the enzyme Proteinase-K to Enterocins. The results showed that the antibacterial activity of Enterocins was lost after the addition of these proteolytic enzymes. This is because bacteriocin is a protein was denatured by the proteinase-K enzyme (Avaiyarasi et al., 2016).

The opposite result shows that the antibiotic (control), which is a non-protein compound, still has antibacterial potential (able to inhibit growth) of the three tested bacteria (Figure 3). These results further strengthen the evidence that the metabolites produced by *E. faecalis* K2B1 are bacteriocins. Bacteriocin is a protein that results from the expression of a bacterial gene, so its effectiveness for bacteriocin production is highly dependent on the conditions that allow the expression of the gene concerned (Umniyati et al., 2006). Bacteriocin production can be carried out in a liquid substrate by observing the optimum production conditions.

Identification of gene encoding enterocins using ent A, B, P, and L50A / B. The amplification results show that only EntL50A/B can be amplified, with a length of 86 bp. Research Batdorj et al. (2006) found EntL50A/B measuring 98 bp, Ogaki et al. (2016) measuring 274 bp and Braiek et al. (2017) measuring 135. Subsequently conducted BLASTX and EntL50A/B sequences showed that the bacteriocin gene sequence *E. faecalis* K2B1 had 94% similar to the *hypothetical protein* EB34_00789 *E. faecalis* on GenBank with *accession number* RBR60004.1 (Figure 3).

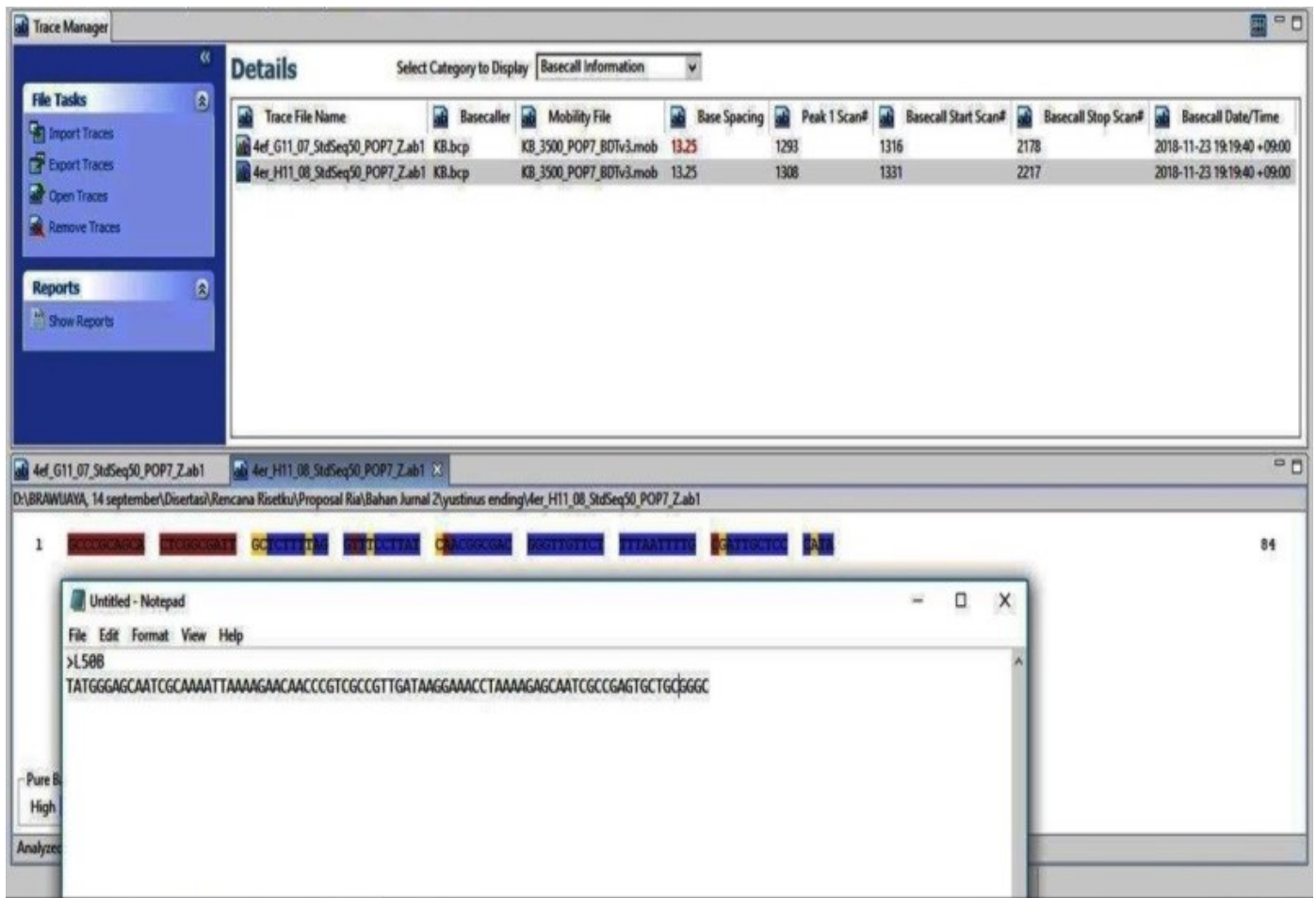


Figure 5. The gene sequence for Enterocins EntL50A / B

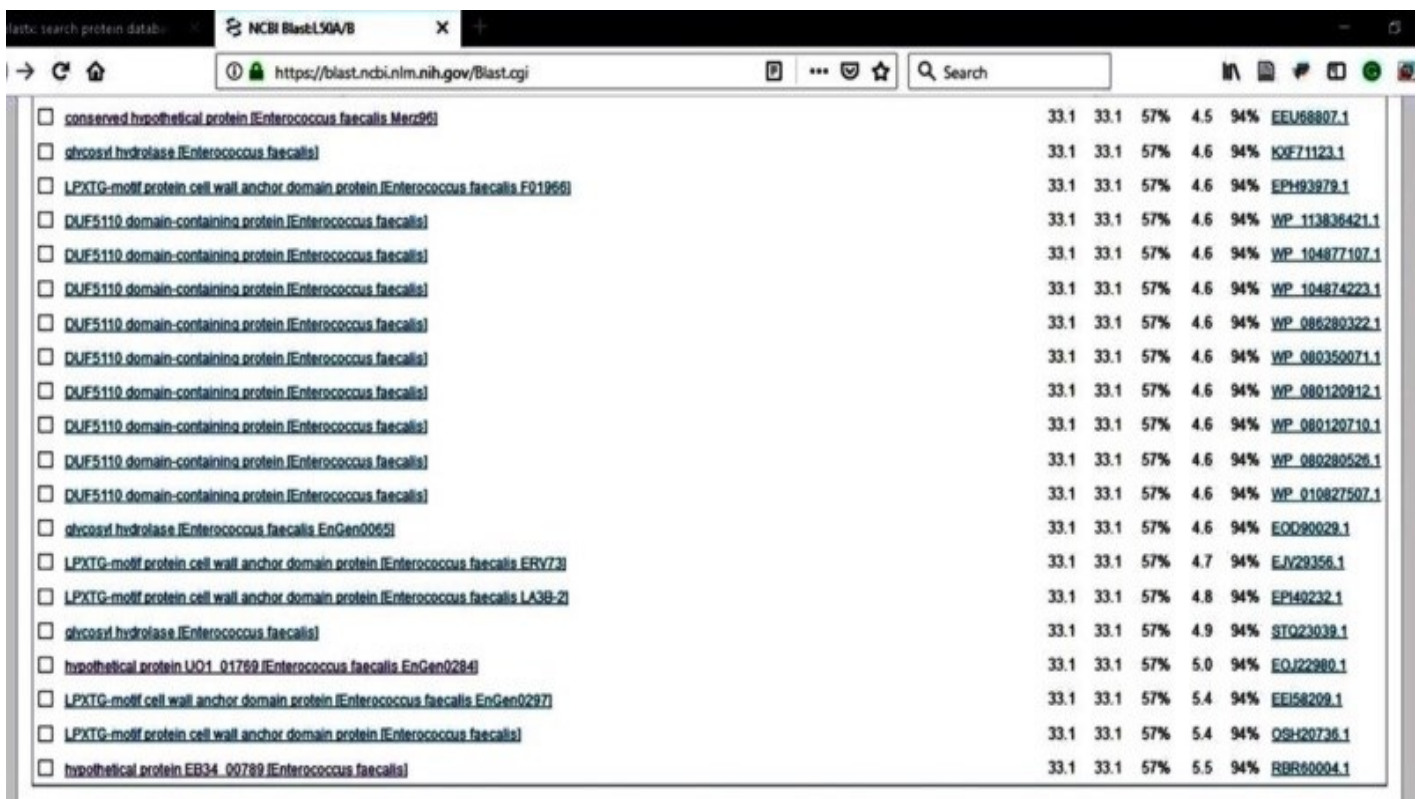


Figure 6. Results of the BLASTx gene encoding Enterocins (EntL50A / B)

Hypothetical protein is a precursor to enterocins, namely the structural gene that encodes prabacteriocin (Todorov, 2009). Similar results were found by Hosseini et al. (2009) used EntA, EntP, EntL50A/B, but only the amplified EntP and showed 95% homology with the *hypothetical protein* EfaeDRAFT 0811 on GenBank with the accession number ZP_00604146. This probably due the *E. faecalis* K2B1 isolate has undergone a mutation.

Some Enterococcus strains that have mutated cannot have the *ABC transporter gene* or *accessory protein gene* and only carry the *structural genes* coding for pra bacteriocins (Ogaki et al., 2016). However, these propeptides are able to fight pathogenic bacteria and have an important role in response to stress by bile salts so that they can survive in environmental conditions containing bile salts (Braïek et al., 2017; Hosseini et al., 2009; Ogaki et al., 2016; Toğay et al., 2016).

The formation of bacteriocins involves several genes that are regulated by operons, namely *genes encoding peptide precursors* (structural genes coding for pra bacteriocins), *immunity genes* (genes for resistance to bacteriocin activity), *ABC transporter genes* (membrane transport) which play a role in the excretion of bacteriocins to pass through membranes, and induction/accessory protein-coding genes that play a role in bacteriocin processing (Drider et al., 2006; Zacharof & Lovitt, 2012).

The detection of EntA, EntP, EntLB50A/B has also been carried out by Sarra et al. (2013) against *E. faecium* and Braïek et al. (2017) against *E. lactis*. In the study, only EntP was amplified in *E. faecium* with a size of 130 bp and *E. lactis* with a size of 139 bp. Saelim et al. (2012) stated that when Enterococcus has the entA gene structure, it does not mean that it produces Enterocins A, to ensure the production of Enterocins A, further purification of the antimicrobial peptide is required.

The detection of EntA and EntB against *E. faecium* was also carried out by (Banwo et al., 2013). In that study, only EntA could be amplified with a length of 140 bp. Several studies have stated that EntB is often detected when EntA is present, but this does not occur when a mutation, so the entB gene is no longer found in that species. The Ent50A/B gene from *E. faecium* LCW 44 was discovered by Vimont et al. (2017), has a size of 275 bp and has a broad spectrum (inhibits *Lysteria monocytogenes* and *Staphylococcus aureus*). Izquierdo et al. (2008) found Ent50A / B from *E. faecium* IT62 to have a broad spectrum against Gram-negative and positive bacteria. This means EntL50A/B *E. faecalis* K2B1 isolated from Toraja's Belang buffalo milk is very different from the EntL50A/B Enterococcus isolate several previous researchers have found that.

The more genes that encode Enterocins, the better their activity as an antimicrobial because more Enterocins are produced. However research by Alang et al. (2020)

previously found that *E. faecalis* K2B1 was also able to inhibit Gram-negative (*Staphylococcus aureus* and *Bacillus cereus*) and also Gram-negative bacteria (*Pseudomonas aeruginosa*), it means that it was having a broad spectrum of inhibition. Expression of the Enterocins gene as an antimicrobial is not only influenced by the coding genes, but also by other factors such as environmental conditions and genetic mechanisms such as operons.

Conclusions

Based on the results of the study, it can be concluded that ent A, B, P encoding enterocins in *E. faecalis* K2B1 isolate cannot be amplified, and only entL50A / B can be amplified, has a size of 86 bp and is similar to hypothetical protein by 94%.

Declaration statement

The authors declare that they have no known competing financial interests or personal relationships that could have influenced the work reported in this paper.

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