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Isolasi Dan Identifikasi Bakteri Asam Laktat Penghasil Bakteriosin Dari *Urutan*, Sosis Fermentasi Tradisional Bali

Isolation And Identification Of Bacteriocin Producing Lactic Acid Bacteria From *Urutan*, Balinese Traditional Fermented Sausage

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INTISARI

Bakteri asam laktat (BAL) merupakan kelompok bakteri utama dalam *urutan* yang memegang peranan penting dalam proses fermentasi serta mencegah pertumbuhan atau membunuh bakteri patogen. *Urutan* adalah sosis khas Bali yang diproduksi secara tradisional dan terfermentasi secara alami. Penelitian ini bertujuan untuk melakukan isolasi dan identifikasi BAL yang dapat menghasilkan bakteriosin dari *urutan*. *Urutan* yang digunakan pada penelitian ini diperoleh dari beberapa pedagang di daerah Baturiti dan Mengwi, Bali. Sebanyak 14 isolat bakteri yang menghambat bakteri indikator pada prosedur *overlay* agar, diisolasi serta dikonfirmasi sebagai BAL yang ditunjukkan dengan hasil uji katalase negatif dan Gram positif. Uji konfirmasi bakteriosin dilakukan dengan metode disk difusi menggunakan bakteri indikator *Escherichia coli* ATCC8739, *Staphylococcus aureus* ATCC25923, *Enterococcus faecalis* ATCC29212 dan *Lactobacillus plantarum* E12.1. Bakteriosin kasar *Lactobacillus* sp. J2 dan J6 menunjukkan zona hambat terhadap pertumbuhan *L. plantarum* E12.1 dan *E. coli* ATCC8739. Bakteriosin kasar *Lactobacillus* sp. J6 juga menghambat pertumbuhan *E. faecalis* ATCC29212. Analisis sekuen gen 16S rRNA menunjukkan *Lactobacillus* sp. J2 dan J6 teridentifikasi sebagai *Lactobacillus plantarum* dengan kemiripan sekuen *Lactobacillus* sp. J2 mencapai 99,39% dan kemiripan sekuen *Lactobacillus* sp. J6 mencapai 99,35%. Dari hasil penelitian ini disimpulkan bahwa *L. plantarum* J2 dan *L. plantarum* J6 adalah BAL penghasil bakteriosin pada *urutan*.

Kata kunci: Bakteri Asam Laktat, Bakteriosin, *Lactobacillus plantarum*, *Urutan*

ABSTRACT

Lactic acid bacteria (LAB) are the main bacterial group in the *urutan* which play an important role in the fermentation process and inhibit the growth of pathogenic bacteria. *Urutan* is a Balinese sausage, that is traditionally produced and naturally fermented. The objective of this study was to isolate and identify LAB that can produce bacteriocins from the *urutans*. The *urutans* used in this study were purchased from several traders in the Baturiti and Mengwi region, Bali. A total of 14 bacterial isolates that inhibited indicator bacterial growth on overlay agar procedure,

were isolated and confirmed as LAB by Gram-positive bacteria and negative results on the catalase test. Bacteriocin confirmation test was carried out by a disk diffusion method using *Escherichia coli* ATCC8739, *Staphylococcus aureus* ATCC25923, *Enterococcus faecalis* ATCC29212 and *Lactobacillus plantarum* E12.1 as indicator bacteria. Crude bacteriocin from *Lactobacillus* sp. J2 and J6 inhibited the growth of *L. plantarum* E12.1 and *E. coli* ATCC8739. Crude bacteriocin of *Lactobacillus* sp. J6 also inhibited the growth of *E. faecalis* ATCC29212. Analysis of 16S rRNA gene sequences showed that *Lactobacillus* sp. J2 and J6 were identified as *Lactobacillus plantarum* with the similarity of the *Lactobacillus* sp. J2 sequence is 99.39% and the similarity of the *Lactobacillus* sp. J6 sequence is 99.35%. The conclusion of this study is *L. plantarum* J2 and *L. plantarum* J6 were LAB which produced bacteriocin in the *urutan*.

Keyword: Lactic Acid Bacteria, Bacteriocin, *Lactobacillus plantarum*, *Urutan*

INTRODUCTION

Lactic acid bacteria (LAB) is an important group of microorganisms involved in the production of various fermented food. LAB could produce various compounds with antagonistic activity against other microbes (Perez et al., 2014). The ability to inhibit the growth of spoilage microorganisms and pathogenic bacteria contribute to the maintenance of hygienic and quality of the product or host health (Kormin et al., 2001). Those compounds include organic acids, H₂O₂, CO₂, diacetyl, acetaldehyde, and bacteriocins (Yang et al., 2012).

Bacteriocins are proteins or complex proteins biologically active with antimicrobial action against other bacteria, principally closely related species (Deraz et al (2005) in Parada et al (2007). The bacteriocins produced by LAB are classified as Generally Recognized as Safe (GRAS) by the Food and Drug Administration (FDA). The bacteriocins also have superior properties such as tolerance to high temperatures, relatively wide antimicrobial spectrum but most at closed related bacteria, activity over a wide pH range, colorless, odorless, and tasteless (Perez et al., 2014).

Due to the advantages, bacteriocins have caught the attention of food researcher and industries as natural food bio preservatives to replace the use of chemical-based food preservatives that according to Bali et al., (2011) could cause undesirable side effects such as alteration in the constituents, nutritional and organoleptic properties of the food and toxic effects on human health.

Bacteriocin as a biopreservative agent is not widely available and relatively expensive. On the other hand, Indonesia has various types of fermented food which are natural sources of LAB. One of the naturally fermented foods that has not been much studied is *urutan*, a traditional fermented sausage from Bali.

Antara, et al. (2002) conducted identification and succession of LAB during the fermentation process of *urutan*. From this study, six LAB species were obtained, there are *Lactobacillus plantarum*, *Lactobacillus farciminis*, *Lactobacillus fermentum*, *Lactobacillus hilgardii*, *Pediococcus acidilactici*, and *Pediococcus pentosaceus*. Aryanta (2013) also isolated *Pediococcus cerevisiae* from *urutans* that were allowed to ferment naturally. These studies showed that several LAB species grew during the fermentation process of *urutan*, and they are possibly bacteriocin-producing LAB. However, the capability of LAB isolates to produce bacteriocins were not tested in these studies. Therefore, in this study authors aim to obtain bacteriocin-producing LAB isolates from *urutans* and to identify the species on the basis of their 16S rDNA sequences compared with those deposited at the GeneBank.

MATERIALS AND METHODS

Bacterial Strains and Culture Media

Escherichia coli ATCC8739, *Staphylococcus aureus* ATCC25923, and *Enterococcus faecalis* ATCC29212 were obtained from the Microbiology Laboratory of the Faculty of Medicine, Udayana University,

while *Lactobacillus plantarum* E12.1 was obtained from the author's collection, were used as indicator bacteria in the assays. Blood agar plate supplemented with 5%-defibrinated sheep blood was used to culture *Escherichia coli* ATCC8739, *Staphylococcus aureus* ATCC25923, and *Enterococcus faecalis* ATCC29212. On the other hands, *Lactobacillus plantarum* E12.1 was cultured by using MRS (*De Man, Rogosa, and Sharpe*) agar.

Isolation and Screening for Antibacterial-Producing LAB

Screening for antibacterial-producing LAB was carried out by the overlay method, as specified in Subagiyo et al. (2016). A quantity of 10 g *urutans* were homogenized in 90 mL of sterile saline solution (0.85% NaCl) and diluted in a series of multiples of ten. From the 10^{-5} and 10^{-6} dilution rate, each as much 0.1 mL were spread over two different MRS agar plates and incubated at 37°C for 48 hours under anaerobic conditions. After 48 hours, the bacterial colonies that grew on the first plate was overlaid with 10 mL MRS soft agar which added with 100 µL suspension of 0.5 McFarland *E. faecalis* ATCC29212, while the second plate was overlaid with 10 mL MRS soft agar added with 100 µL suspension of 0.5 McFarland *L. plantarum* E12.1. The plates were then incubated at 37°C for 48 hours under anaerobic conditions to produce inhibition zones. Bacterial colonies with clear zones were collected and examined with the catalase test and Gram stain test to ensure that the isolates were LAB.

Confirmation Test for Bacteriocin-Producing LAB

Confirmation tests were carried out using the disk diffusion method. The indicator bacteria were *S. aureus* ATCC25923, *E. coli* ATCC8739, *E. faecalis* ATCC29212, and *L. plantarum* E12.1. Production of crude bacteriocins and inhibition test were conducted according to Sari et al. (2016). Antibacterial-producing LAB colonies were cultivated in MRS broth and incubated at 37°C for 18-20 hours under anaerobic conditions. This was

followed by centrifugation at 8,000 rpm for 15 minutes to separate the cells from the supernatant. The supernatant was neutralized by adding 1M NaOH until the pH reached 6-7, then filtered with a 0.22 milipore membrane to obtain the neutral cell-free supernatant (crude bacteriocin). A total of 20 µL of crude bacteriocin were dropped on a blank disc until completely absorbed. The disc was placed on Nutrient Agar (NA) which had been rubbed with 0.5 McFarland indicator bacterial suspension. After incubation at 37°C for 24 hours, the diameter of the inhibition zone (clear zone as shown in figure 1) was measured. The test was carried out in triplicates. The average diameter of the inhibition zones was interpreted according to Pan et al. (2009) as follows: 0-3 mm is weak, 3-6 mm is moderate, and > 6 mm is strong.

Identification of Bacteriocin-Producing LAB Isolates using 16S rRNA gene sequencing

The identification was carried out by amplification of the 16S rRNA gene using the PCR method, followed by sequencing. DNAs were isolated using the High Pure PCR Template Preparation Kit (Roche Molecular Biochemicals, IN, USA) according to the manufacturer's instruction. The primer design and PCR procedure were conducted according to Arief et al. (2015). The universal primers 9F (5'-GAG TTT GAT CCT GGC TCA G-3') and 1541R (5'-AAG GAG GTG ATC CAG CC-3') were used with slight modification on the PCR condition. The PCR formulation consisted of 15 µL of GoTaq® Green Master Mix, 2,4 µL of 10 µM primers, 3 µL of template DNA, and H₂O until the total volume of 30 µL was reached.

DNAs were amplified using Thermal Cycler (*Biometra*) under the following conditions: pre-denaturation at 95°C for 5 minutes followed by 30 cycles of denaturation at 95°C for 1 minute, annealing at 55°C for 1 minute, extension at 72°C for 1 minute, and elongation at 72°C for 7 minutes. PCR products were electrophoresed on 1% agarose gel containing 0,003% GelRed® (Biotium) and visualized on UV-transilluminator. The

expected DNA was then sequenced to identify the nucleotide base sequence (First Base, Malaysia).

The DNA sequence was edited with the Biological Sequence Alignment Editor (BioEdit) then presented in the form of Fasta files and aligned with the nucleotides of known bacteria strains on the Basic Alignment Search Tool (BLAST) available on the website of <https://blast.ncbi.nlm.nih.gov/Blast.cgi>.

RESULTS AND DISCUSSION

Isolation for Bacteriocin-Producing LAB

LAB was isolated from five samples of indigenous fermented *urutans*. A total of 14 colonies with clear zones that inhibited the growth of indicator bacteria were isolated in this study. Those 14 isolates confirmed as LAB based on Gram staining result that showed as Gram-positive bacilli or cocci and negative catalase test result. These results were in line with those reported by Todar (2012) with LAB isolate characteristics of Gram-positive cocci, coccobacilli, or bacilli, non-forming spores, generally anaerobic, and lack of catalase enzymes.

LAB could produce various compounds with antagonistic activity against other microbes. Those compounds include organic acids, H₂O₂, CO₂, diacetyl, acetaldehyde, and bacteriocins (Yang et al., 2012). To confirm that the inhibition zones were due to bacteriocins affect, the confirmation test was carried out using the neutralized supernatant (crude bacteriocins). Crude bacteriocins of 14 isolates were tested, only two isolates named J2 and J6 showed inhibition zones against indicator bacteria (Table 1).

Neutralization of culture supernatants resulted in the loss of organic acid activity. According to Daeschel (1989), organic acids are relatively more abundantly produced than other antimicrobial compounds (Artha, 2016). Hu et al. (2019) also stated that the key to strong antimicrobial activity in three strains of *L. plantarum* against *S.aureus*, *E.coli*, and Salmonella is the organic acid. Therefore, after the supernatant was neutralized, LAB isolates

with no ability to produce bacteriocins lost their activity.

Table 1. Results of Confirmation Test for Bacteriocin-Producing LAB

Tested Bacteria	Inhibition Zone			
	J2		J6	
	Ø (mm)	Int	Ø (mm)	Int
<i>E. coli</i> ATCC 8739	2,53	+	3,93	++
<i>S. aureus</i> ATCC 25923	0	-	0	-
<i>L. plantarum</i> E12.1	5,07	++	10,27	+++
<i>E. faecalis</i> ATCC 29212	0	-	2,7	+

Note: Ø = diameter, Int = Interpretation, (-) = no inhibition zone formed, (+) = weak, (++) = moderate, (+++) = strong

Bacteriocins are classified into several types. Classical type bacteriocins are active only against homologous species, while the second type can show inhibitory activity against a broad range of Gram-positive microorganisms (Parada et al., 2007). In this study, the bacteriocins produced by *Lactobacillus* sp. J2 and J6 showed moderate (5,07 mm) to strong (10,27 mm) inhibition zones against *L. plantarum* E12.1 and weak (2,7 mm) inhibition zones against *E. faecalis* ATCC29212 by *Lactobacillus* sp. J6.

Similar results are shown by Romadhon and Margino (2012), where they found that the bacteriocins produced by two LAB isolates from the tiger shrimp intestine effectively inhibited the growth of *Pediococcus accidilactici* LB 42. Both isolates were identified as *Pediococcus accidilactici*. Kormin et al. (2001) found that *L. plantarum* from tempeh inhibited the growth of *Enterococcus faecalis* N-I-103. Bacteriocins produced by *Lactobacillus* sp. J2 and J6 did not show inhibitory activity against the Gram-positive *S. aureus*, but showed weak to moderate inhibitory activity against the Gram-negative *E. coli*. Similar results were also reported by Sari et al.

(2016), in which the bacteriocins produced by *L. plantarum* isolated from the Ce Hun Tiau drink did not show an inhibition zone against *S. aureus* but they inhibited the growth of *E. coli*.



Figure 1. Crude bacteriocins of *Lactobacillus* sp. J2 and J6 formed inhibition zones against *L. plantarum* E 12.1 (source: personal documentation, 2020).

The target of bacteriocins is the cytoplasmic membrane. *Lipopolysaccharide* (LPS) outside the membrane layer of Gram-negative bacteria protects the bacteria from attack by bacteriocins. Therefore, bacteriocins are generally only active against Gram-positive bacterial cells (Parada et al., 2007 and Bromberg et al., 2004). However, mutations or exposure to stress such as heating, freezing, and thawing could damage the outer membrane, which allows access of the bacteriocin to the cytoplasmic membrane thereby increasing sensitivity to bacteriocins (Bromberg et al., 2004). Different mechanisms of action from bacteriocins were also described, such as changing enzymatic activity, inhibition of spore germination, and inactivation of carrier anions through the formation of selective and non-selective pores (Abee, 1995; Martinez and De Mantis, 2006 in Parada et al., 2007).

Bacteriocin's inhibitory activity against Gram-negative bacteria was also shown in several other studies. Bromberg (2004) found that from 128 bacteriocin-producing LAB strains, 47.6% inhibited the growth of Gram-negative bacteria *Pseudomonas* sp CTC 32, 38.3% inhibited *E. coli* ATCC25422, and

37.5% inhibited *Salmonella typhimurium* ATCC14028.

Lade et al. (2006) in Parada et al., (2007) found that *Lactobacillus plantarum* and *Lactobacillus lactis* isolated from vegetable wastes produced bacteriocins which inhibited the growth of *E. coli*. Bacteriocin can be bactericidal or bacteriostatic. Bacteriocin's antibacterial activity is greatly influenced by factors such as environmental conditions and strain of indicator bacterial species used, concentration and purity of the bacteriocin, nutrient content in the medium (Romadhon and Margino, 2012; Sari, et al., 2018), and experimental environment (Cintas et al., 2001). Stern et al. (2006) in their study on *Lactobacillus salivarius* showed that the activity of purified bacteriocin was 89.8% higher than the supernatant from bacterial cultures (Artha, 2016).

Identification of Bacteriocin-Producing LAB Isolate

Identification of *Lactobacillus* sp. J2 and J6 isolates were carried out by amplification of the 16S gene using 9F and 1541R primers. The PCR products were \pm 1500 bp (Figure 2) then sequenced, and the results were aligned with the database at the Gene Bank. The size of the *Lactobacillus* sp. J2 and J6 16S rRNA gene sequences was 1481 bp and 1494 bp, respectively. Aligned of the *Lactobacillus* sp. J2 sequence on BLAST showed 99.39% similarity with *Lactobacillus plantarum* strain 33X, while the *Lactobacillus* sp. J6 sequence showed 99.35% similarity with *Lactobacillus plantarum* strain 7232 (multiple sequences alignment/MSA showed in figure 3). Therefore, both *Lactobacillus* sp. J2 and J6 isolates belong to species of *Lactobacillus plantarum*. *Lactobacillus plantarum* is known as a bacteriocin-producing LAB species. According to Baltasar (2015), bacteriocin-producing *L. plantarum* strains have been isolated from various vegetable and animal products such as cereals, wines, meats, and dairy products. Bacteriocins produced by *L. plantarum* are generally indicated as plantaricin which are classified into two classes, namely bacteriocin

class I (lantibiotic) and bacteriocin class II (non-lantibiotic). These two strains were in the same clade as the J2 isolate, which indicated that the three strains of bacteria came from the

same bacteria. As a comparison, J2 and J6 isolates were also aligned with several strains of *L. plantarum*, *Lactobacillus* species, and other LAB genera (Figure 4)

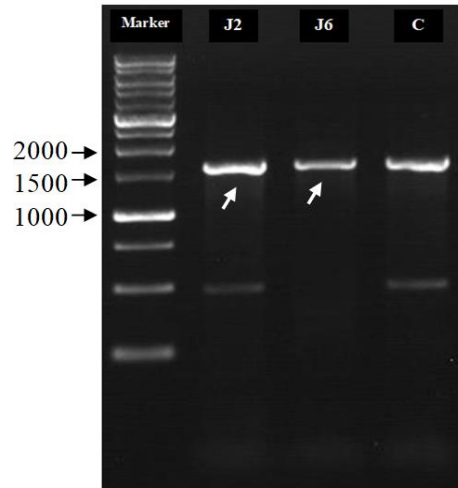


Figure 2. Visualization of PCR products. Lane 1: J2 isolate, lane 2: J6 isolate, lane 3: positive control *L. plantarum* E12.1. (source: Personal documentation, 2020).

Phylogenetic tree analysis showed that the J6 isolate was closely related to *L. plantarum* E 12.1.

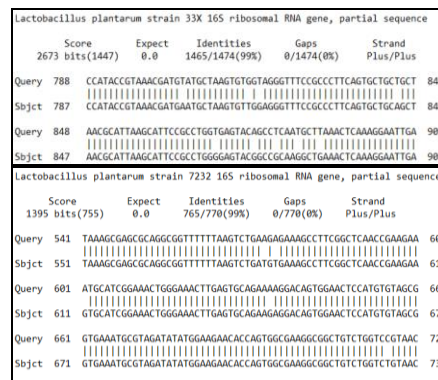


Figure 3. MSA of *L. plantarum* J2 with *L. plantarum* 33X (up) and MSA of *L. plantarum* J6 with *L. plantarum* 7232 (down) (source: Personal doc., 2020).

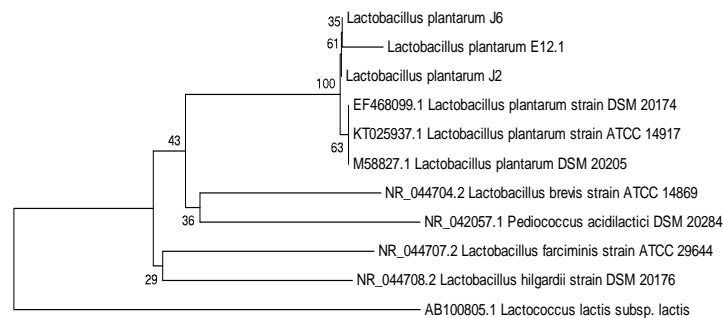


Figure 4. Phylogenetic tree of 16S rDNA sequences of *L. plantarum* J2 and J6 isolates using the neighbor-joining method with 1000 replications bootstrap test.

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CONCLUSION

Two LAB isolates (J2 and J6) with the ability to produced bacteriocins were successfully isolated in this study. Molecular identification of these isolates showed that they both belong to *Lactobacillus plantarum*. Both strains are potential for use as bio preservative agents, although further studies are still needed. Purification and characterization of the bacteriocins are also needed to be conducted in further studies.

REFERENCES

- Antara, et al. 2002. Identification and succession of lactic acid bacteria during fermentation of urutan, a Balinese indigenous fermented sausage. *World Journal of Microbiology & Biotechnology* 18:255-265.
- Arief, Irma Isnafia, et al. 2015. Identification and probiotic characteristics of lactic acid bacteria isolated from Indonesian local beef. *Asian Journal of Animal Science* 9 (1):25-36.
- Artha, Melinda Sanggu. 2016. Pertumbuhan bakteri asam laktat *Enterococcus faecium* sebagai penghasil bakteriosin pada kondisi aerob dan anaerob (Bachelor's Thesis). Mataram: University of Mataram.
- Aryanta, I.W.R. 2013. Microbiology and processing of traditional foods in Bali, Indonesia. Mikrobiologi Pangan dan Pakan. Denpasar: Udayana University Press.
- Bali, V.P., Panesar, S., and Bera, M.B. 2011. Isolation, screening, and evaluation of antimicrobial activity of potential bactericin producing lactic acid bacteria isolate. *Microbiology Journal* 1(3):113-119.
- Baltasar, Mayo Ana, Belén Flórez. 2015. Lactic acid bacteria: *Lactobacillus* spp.: *Lactobacillus plantarum*. Elsevier Reference Module in Food Science. Available from: <https://www.sciencedirect.com>.
- Bromberg, Renata, Izildinha Mreno, Cintia L. Zaganini, Roberta R. Delboni, and Josiane de Oliveira. 2004. Isolation of bacteriocin-producing lactic acid bacteria from meat and meat products and its spectrum of inhibitory activity. *Brazilian Journal of Microbiology* 35: 137-144.
- Cintas, L.M., Herranz, C., Hernandez, P.E., Casaus, M.P. and Nes, L. F. 2001. Review: Bacteriocins of Lactic Acid Bacteria. *Food sci. Tech. Int.*, 7, 281-305.
- Hu, Chang Hui, Li Quan Ren, Ying Zhou, and Bang Ce Ye. 2019. Characterization of antimicrobial activity of three *Lactobacillus plantarum* strain isolated from Chinese traditional dairy food. *Food Science and Nutrition* 2019;7.
- Kormin, S., et al. 2001. Bacteriocin-producing lactic acid bacteria isolated from traditional fermented food. *Malaysian Journal of Medical Sciences*, Vol.8 No.1 (63-68).
- Pan,X.,Chen F.,Wu T.,Tang H., dan Zhao Z. 2009. The Acid, Bile Tolerance and Antimicrobial Property of *Lactobacillus acidophilus* NIT. *Jurnal Food Control*. 20 : 598-602.
- Parada, Jose Luis, Carolina R. Caron, Adriane B.P. Medeiros, ann Carlos R. Soccol. 2007. Bacteriocins from lactic acid bacteria: purification, properties, and use as biopreservatives. *Brazilian Archives of Biology and Technology* Vol 50, n. 3 pp.521-542.
- Perez, Rodney H., Takeshi Zendo, and Kenji Sonomoto. 2014. Novel bacteriocins from lactic acid bacteria (LAB): various structures and applications. *Microbial Cell Factories*,13 (Suppl 1): S3.
- Romadhon, Subagiyo, and Sebastian Margino. 2012. Isolasi dan karakterisasi bakteri asam laktat dari usus udang penghasil bakteriosin sebagai agen antibakteria pada

- produk-produk hasil perikanan. *Jurnal Sainstek Perikanan* Vol.8 No.1.
- Sari, Novi Permata, Rafika Sari, and Eka Kartika Untari. 2018. Antibacterial activity test of bacteriocin from *Lactobacillus brevis*, *Lactobacillus casei*, and *Lactobacillus plantarum* against gram-positive pathogenic bacteria. *Journal of Tropical Biodiversity and Biotechnology* Vol.3 85-91.
- Sari, Rafika, Lia Deslianri, and Pratiwi Apridamayanti. 2016. Skrining aktivitas antibakteri bakteriosin dari minuman Ce Hun Tiau. *Pharm Sci Res Reviews* 4:124-140.
- Subagiyo Subagiyo, Sebastian Margino, Triyanto Triyanto, Ria Azizah Tri Nuraini, Wilis Ari Setyati, and Rini Pramesti. 2016. Metode sederhana dan cepat untuk skrining bakteri asam laktat penghasil bakteriosin (antimicrobial peptide) dari intestinum ikan dan udang. *Buletin Oseanografi Marina* Vol 5 No 2 :97 – 100.
- Todar, Kenneth. 2012. Todar's online textbook of bacteriology: lactic acid bacteria. www.textbookofbacteriology.net. Accessed 3/7/2019.
- Yang,En, Lihua Fan, Yueming Jiang, Craig Doucette, dan Sherry Fillmore. Antimicrobial Activity of Bacteriocin-Producing Lactic Acid Bacteria Islated From Cheeses and Yogurts.*AMB Express* 2012, 2:4