Indonesian Journal of Biotechnology

VOLUME 24(1), 2019, 1-7 | RESEARCH ARTICLE

The characterization of bacteriocins produced by *Lactobacillus plantarum* strains isolated from traditional fermented foods in Indonesia and the detection of its plantaricin-encoding genes

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SUBMITTED 13 January 2019 **REVISED** 11 April 2019 **ACCEPTED** 23 May 2019

ABSTRACT *Lactobacillus plantarum* is widely found in either anaerobic plant matter or fermented foods, and it has been recognized as producing antimicrobial bacteriocins. This study aimed to characterize the antimicrobial bacteriocins of *L. plantarum* and detect its genes that encode plantaricins. Samples were isolated from traditional fermented foods from Indonesia. Antimicrobial activity was evaluated using the agar diffusion assay procedure. The titration method applied the maximum amounts of lactic acid at 1054 mg/mL and hydrogen peroxide at 3.85 mg/mL. Based on the results, the supernatant of the *L. plantarum* strains appeared to have a broad spectrum of antimicrobial activity against pathogens, which would be active at pH 2.0–12.0 and stable temperature. In addition, almost all of the *L. plantarum* strains contained plantaricin-encoding genes (e.g. plnA, plnF, plnJK, and plnW), which were grouped into one cluster as indicated by phylogenetic analysis. Therefore, this study discovered clear evidence of the potential of some *L. plantarum* strains to act as antimicrobial agents.

KEYWORDS antimicrobial agent; bacteriocin; *Lactobacillus plantarum*; plantaricin genes

1. Introduction

Lactobacillus plantarum is classified as a lactic acid bacterium (LAB) group and Gram-positive bacteria that produces lactic acid as its main fermentation product into the culture medium. Generally recognized as safe (Konings et al. 2000), it is associated with many Indonesian traditional fermented foods such as bekasam (meat fermented food), tapai (fermented glutinous rice), and tempoyak (durian fermented food). These foods have been consumed for centuries, but no investigation has been conducted yet to assess the genes in LAB isolated from them. Bekasam was processed with mixing flesh with 10–20% salt (w/v) and ground, roasted rice, then fermented (in sealed container) for 14 days. While tempoyak durian (*Durio zibethinus*) was made by mixing flesh with 2.5% salt (w/v) and placed in a sealed container to ferment for about 7 days. On the other hand, Tapai ketan was created by steaming ketan (glutinous rice) followed by inoculation with ragi tapai, then fermented for about 1–2 days until an acid-alcoholic taste was achieved (Mustopa 2014). These anaerobic bacteria play an essential role in extending the shelf life of fermented products and are known to produce antimicrobial substances such as organic acids, free fatty acids, hydrogen peroxide and bacteriocin which can inhibit the growth of several microbial (Mustopa 2014). Recently, various bacteriocin produced by *L. plantarum* isolated from fermented foods were investigated for their antimicrobial activity (Mustopa 2014; Xie et al. 2011).

Bacteriocins are ribosome–associated antimicrobial peptides as in a modified or unmodified state synthesized by Gram positive and Gram negative bacteria (Leroy and De Vuyst 2004; Xie et al. 2011). These small peptides have unique characteristics, such as (a) broad antimicrobial activity against the same species or across genera, (b) active peptides containing 20–60 amino acids, (c) specific receptors on the target cell, and (d) biosynthesis genes that involve plasmid (Drider et al. 2006). The bacteriocins produced by *L. plantarum* such as plantaricin A, E, F, J, and K belong to class II bacteriocin while plantaricin W is classified into class I of bacteriocin due to amino acid modification on it peptide (Kjos et al. 2009). In this study, a bacteriocin producing *L. plantarum* isolated from Indonesian fermented foods is identified and characterized, to determine its potential for various applications. Furthermore, the gene cluster encoding bacteriocin was determined.

2. Materials and methods

2.1. Bacterial Strains and growth conditions

Seven strains of *L. plantarum* were assayed for antimicrobial activity (Table 1). It was grown in MRS (De Man,

Rogosa, & Sharpe) broth (Oxoid) medium and incubated at 37°C for 20 h under anaerobic conditions according to the method proposed by Xie et al. (2011), with modifications. Bacterial strains used as indicators in this study and growth condition are shown in Table 1.

2.2. Assay for antimicrobial activities of the lactic acid bacteria isolates

Cell-free culture supernatants for antimicrobial assay was prepared by growing lactic acid bacteria (1%, v/v) into 250 mL MRS medium at 37°C for 20 h. The cells were harvested with centrifuged at 12.000 g, 4°C for 15 min. The antimicrobial activity was determined by agar well diffusion assay. Microbial indicator were grown in a nutrient broth at 37°C overnight (Xie et al. 2011).

2.3. Determination of lactic acid and hydrogen peroxide production by the LAB isolates

Strain isolates of lactic acid bacteria were cultured in 100 mL MRS broth at 37°C temperature for 20 h. Next, 25 mL of broth culture were added 3 drops of phenolphthalein as indicator. Meanwhile, 0.1 N sodium hydroxide was placed in to the burette, which them slowly added into the broth culture until its color changed to pink. In general, each milliliter of sodium hydroxide is equivalent to 90.80 mg of lactic acid (Lelise et al. 2014). Then, 25mL of diluted sulphuric acid was added into the 25 mL broth culture to determine its hydrogen peroxide content.

Titration was carried out with 0.1 N potassium permanganate up to decolourization occur. Each 1 mL of 0.1 N potassium permanganate is equivalent to 1.070 mg of hydrogen peroxide. A decolorization for each sample was regraded as end point (Lelise et al. 2014).

2.4. Sensitivity of bacteriocin to temperature, pH, enzymes, and surfactant

All strains was grown in MRS medium for 20 h at 37°C and cell were harvested with centrifugated 12.000g at 4°C for 15 min. The effect of pH on the activity of plantaricin was tested by adjusting cell-free supernatants from pH 2.0 to 12.0 (at increments of two pH units) with sterile 0.1M sodium hydroxide or 0.1M hydrochloric acid. After incubation for 2 h at 37°C, all the samples pH were readjusted to pH 6.0. The effect of temperature on plantaricin activity was tested by incubating cell-free supernatants (adjusted to pH 6.0) at 40°C, 60°C, 80°C, and 100°C for 10, 30 and 60 minutes respectively according to the method proposed by Teixeira et al. (2013) with modification.

Bacteriocin activity was also assayed after treatment at 121°C for 15 min. Cell-free supernatant was incubated for 2 h in the presence of 1 mg/mL proteinase K (Invitrogen), catalase (Sigma), lyzosyme (Sigma), pepsin (Sigma), SDS (Biobasic), urea (Thermo), EDTA (Bio-rad, USA), PMSF (Sigma), and 1% triton-x (MP Biomedicals). All antimicrobial activity was tested using the well-diffusion method (Teixeira et al. 2013).

2.5. Isolation of genomic DNA and PCR conditions

The total genomic DNA of *L. plantarum* strains was isolated in small scale, 5 mL culture in MRS broth (Oxoid) grown at 37°C overnight. Bacterial cells were collected by centrifugation at 6.000g for 10 min. The genomic or chromosome DNA was obtained according to the method of Zhu et al. (2013) with modification. The pellet was resuspended with 500 µL TE buffer (10 mM Tris–HCl pH 8.0 , 1 mM EDTA) containing 60 mg/mL lysozyme and then incubated at 37°C for 1 h. After incubation, 200 µL of 10% sodium dodecyl sulfate, 100 µL of 5 M sodium chloride and 80 µL of 10% CTAB were added. The mixture was then incubated at 68°C for 30 min and an equal amount of chloroform (1:1 v/v) was added. Centrifugation was conducted at 13.000g for 10 min. The supernatant was collected and 1:1 (v/v) ethanol was added and then centrifuged at 13.000g for 10 min. DNA was dissolved in TE buffer containing 10 µg/mL RNAse (Zhu et al. 1993). Chromosomes DNA were used as templates to detect plan-

TABLE 1 Bacterial strains used in this study.

Purpose for use and strains name	Source	Growth conditions
Potential bacteriocin	Strains	Producing
L. plantarum S12	Bekasam (meat fermented food)	MRS broth at 37°C
L. plantarum S14	Bekasam (meat fermented food)	MRS broth at 37°C
L. plantarum S31	Bekasam (meat fermented food)	MRS broth at 37°C
L. plantarum S34	Bekasam (meat fermented food)	MRS broth at 37°C
L. plantarum T3	Tapai (fermented glutinous rice)	MRS broth at 37°C
L. plantarum T8	Tapai (fermented glutinous rice)	MRS broth at 37°C
L. plantarum U10	Tempoyak (durian fermented food)	MRS broth at 37°C
Indicator strains		
Bacillus subtilis ATCC 19659	–	Nutrient broth at 37°C
Salmonella typhi ATCC 25241	–	Nutrient broth at 37°C
Staphylococcus aureus ATCC 6538	–	Nutrient broth at 37°C
Pseudomonas aeruginosa ATCC15442	–	Nutrient broth at 37°C

TABLE 2 Primers used for detection of genes plantaricin.

Target gene	Forward primer (5'–3')	Reverse primer (5'–3')	T†	bp‡	Reference
plnA	ATTTCATGGTGATTCACGTTTAAATT	CTTACGCCATCTATACG	54	300	This study
plnEF	GGTGGTTTTAATCGGGGCGG	ACGGGGTTGTTGGGGGAGGC	53	285	Cho et al. (2010)
plnJk	ACGGGGTTGTTGGGGGAGGC	TTATAATCCCTTGAACCACC	53	267	Cho et al. (2010)
plnF	GACTGGATCCATGAAAAAATTTCCTAGTTTT	GATCAAGCTTCTATCCGGATGAATCCTC	58.5	159	This study
plnW	GATCAGCCACGATACCAAC	CTAAAGAAAAAGCCCCTGAAAC	58.5	750	Sáenz et al. (2009)
plnAC	ATTTCATGGTGATTCACGTTTAAATT	AAATTGAACATATGGGTGCTTTAAATT	55	2000	This study

† Annealing temperature (°C).
‡ Amplicon size.

taricin genes with specific primers used to amplify genes in plantaricin locus showed in Table 2. The PCR products were separated by electrophoresis using 1% (w/v) agarose gel, which was stained with ethidium bromide and visualized using UV light source (Sukmarini et al. 2014).

2.6. Amplification of plantaricin genes by PCR

Plantaricin genes were amplified in 10 µL volumes each containing 100 ng template DNA, 1 unit/µL Taq DNA polymerase 0.05 µL, 10× buffer 1 µL, 10 mM dNTP 0.2 µL, 0.1 µL forward primer, reverse primer, and their annealing temperatures in this study are listed in Table 2. The PCR reaction were performed with an initial denaturation step at 94°C for 3 min, followed by 35 cycles of 94°C for 30 s, primer annealing temperature for 30 s, and 72°C extension for 30 s, followed by a final extension step at 72°C for 3 min. PCR products were separated by electrophoresis using 1% (w/v) agarose gel, which was stained with ethidium bromide and visualized using UV light source.

2.7. DNA sequencing and phylogenetic analysis

The PCR product was sequenced by 1st BASE company service. Similarity searches with sequences were performed by online BLAST analysis (http://blast.ncbi.nlm.gov/Blast.cgi). For phylogenetic analysis, sequences were aligned by using the CLUSTAL X Software (Thompson et al. 1997). A phylogenetic tree was constructed by the neighbor-joining method and the *Streptococcus pyogenes* strain was used as an outgroup. The stability of the relationship was assessed by bootstrap resampling. Bootstrap analysis was performed for 1000 trials in accordance to clustal X program (Saitou and Nei 1987).

3. Results and discussion

3.1. Screening of potential antimicrobial lactic acid bacteria

The agar well-diffusion assay procedure was used to investigate the antimicrobial activity of seven lactic acid bacteria strains isolated from traditional fermented foods in Indonesia. Based on the results of this experiment, these isolates showed antimicrobial activity against at least bacterial indicators, which was presumed to be attributable as bacteriocin-like substances (BLS). The BLS were determined after pH level was neutralized to medium level and hydrogen peroxide was eliminated from the cell-free supernatants (CFS) (Anupama and Balasingh 2018). In general, almost all of tested LAB were able to kill *Bacillus subtilis* and *Pseudomonas aeroginosa* due to hydrogen peroxide present in medium, which is recognized as a strong oxidizing substance against organic matters. However, U10 isolate showed a broader antimicrobial activity spectrum against *Salmonela typhi*, *Staphylococcus aureus*, *Bacillus subtilis* and *Pseudomonas aeroginosa* due to BLS action. It appeared despite the remaining LAB also that showed similar results after a catalase treatment by using *Bacillus subtilis* and *Pseudomonas aeroginosa* as indicator pathogens. Therefore, this study presumed that various antimicrobial activities attributed to each strain appeared due to the production of bacteriocins or metabolites similar to them into medium as natural defences or an adaptive response to surrounding environment (Zhou et al. 2014). Antimicrobial activities of *L. plantarum* thus would be properly characterized by U10 strain isolate.

3.2. Determination of lactic acid and hydrogen peroxide

A heavy amount of lactic acid was produced during the 20-hour incubation, in which the highest amount was produced by S31 isolate (1054 mg/mL) compared to that of other isolates. On the other hand, the amount of hydrogen peroxide produced during the 20-hour incubation period was also recorded, by which T8 isolate was discovered to produce highest amount of hydrogen peroxide compared to other isolates.

The highest yield of lactic acid (1054 mg/mL) was produced by isolate S31 while the lowest yield of 720.64 mg/mL was produced by isolate S34 and the highest yield of hydrogen peroxide was produced by isolate T8 (3.85mg/mL) while isolate S34 produced the lowest yield (2.35mg/mL). The increase in the production of lactic acid with time has been attributed to lowered pH which permit the growth of LAB. LAB can noted the inhibition of *Bacillus subtilis*, *Salmonella typhi*, *Staphylococcus aureus*, and *Pseudomonas aeroginosa* by hydrogen peroxide of LAB strains which contribute to their inhibitory activity against other microorganisms (González et al. 2015).

3.3. Characterization of the bacteriocin

Plantaricin U10 was inhibitory against a variety of Gram-positive and Gram-negative bacteria. Among the indicator species, the bacteriocin showed activity against *Salmonella typhi*, *Staphylococcus aureus*, *Bacillus subtilis*, and *Pseudomonas aeroginosa*. This result exhibit this BLS has a broad antibacterial activity. Even after treated at 121°C for 5 min (sterilization temperature) decrease antimicrobial from initial activity. This study clearly demonstrated that the BLS obtained from strain U10 is thermostable. Several studies have been reported that the BLS treated at 100°C for 60 min (Okpara et al. 2014) and 121°C for 15 min were stable at this high temperature (Zhou et al. 2014). This heat stability would be a very useful characteristic as antimicrobial peptide thermostable property in biomedicinal it could be used as antimicrobial peptide (Table 3). After incubation for 2 h at pH values from pH 2.0 to 8.0 stable activity but its activity slightly reduced at pH 10.00 and showed activity at pH 12.0 This result showed that the BLS was resistant to acid conditions (Table 4). That agreement with other research on characterized *L. plantarum* that inhibition zone pH treatment stable at range pH 2.0 to 8.0 (Jiang et al. 2017).

The results from enzyme inactivation studies exhibit that antimicrobial activity was lost or unstable after treatment with proteinase-k and pepsin, whereas treatment with catalase, lysozyme did not affect the activity of bacteriocin produced by U10. Furthermore, the antimicrobial activity of bacteriocins produced by U10 strain isolate appeared to not be affected by hydrogen peroxide or acidity, because the activity did not disappear after treatment with catalase/peroxidase or a pH adjustment to 6.0. In addition, non-ionic detergents such as Triton X-100 at a final concentration of 1% did not reveal any significant increase in bacteriocin activity (Table 4). In particular, Triton X-100 was discovered to not be capable of dissociating bacteriocin aggregate or no aggregate at all, causing significant disappearances of antimicrobial activity. However, the anionic detergent SDS increased in bacteriocin activity.

The increase in bacteriocin activity could be attributable to dispersion of the bacteriocin complex thereby releasing more unit for the activity. SDS itself an antibacterial agent thus increased bacteriocin activity shall be obtained (Holo et al. 2001).

3.4. Determination of the genes encoding bacteriocin and phylogenetic relationship

Six genes, which are present in the *pln* locus and are responsible for the synthesis of plantaricin, were searched

TABLE 3 Effect of heat treatment on the antimicrobial activity of U10.

Application	Activity antimicrobial		
	Salmonella typhi	*Staphylococcus aureus*	*Escherichia coli*
CFS*	+++[1]	+++	nd[2]
BLS**	+++	+++	nd
40°C/10 min	+++	+++	+++
40°C/30 min	+++	+++	+++
40°C/60 min	+++	+++	+++
60°C/10 min	+++	+++	+++
60°C/30 min	+++	+++	+++
60°C/60 min	+++	+++	+++
80°C/10 min	+++	+++	+++
80°C/30 min	+++	+++	++[3]
80°C/60 min	++	++	++
100°C/10 min	++	++	+[4]
100°C/30 min	+	++	+
100°C/60 min	+	+	–[5]
121°C/15 min	+	+	–

[1] Inhibitory zone > 10.
[2] Not determined.
[3] Inhibitory zone 8–10 mm.
[4] Inhibitory zone 5–7 mm.
[5] No inhibitory zone.
* CFS: cell-free supernatant from LAB culture.
** BLS: CFC following the pH neutralization and hydrogen peroxide elimination.

TABLE 4 Effect of enzymes, surfactant, and different pH treatment.

Application	Activity antimicrobial		
	Salmonella typhi	*Staphylococcus aureus*	*Escherichia coli*
CFS	+++	+++	+++
BLS	+++	+++	+++
Precipitation	+++	+++	+++
Enzymes			
Proteinase-K (Sigma)	-	-	-
Catalase (Sigma)	+++	+++	+++
Lyzosyme (Sigma)	++	++	++
Pepsin (Sigma)	+	+	+
Surfactant			
SDS	+++	+++	+++
Urea	++	++	++
Triton-X	+++	+++	+++
EDTA	+++	+++	++
PMSF	+++	+++	++
pH			
2	++	+++	++
4	+++	+++	+++
6	+++	+++	+++
8	++	++	++
10	+	++	+
12	+	+	+

TABLE 5 Strains used in this study.

L. plantarum	PlnA	PlnABC	PlnEF	PlnF	plnJK	PlnW
S12	+	−	−	−	−	−
S14	+	+	−	−	−	−
S31	+	−	+	+	+	+
S34	+	−	+	+	+	+
T3	+	−	−	−	+	−
T8	+	−	−	−	+	−
U10	+	−	−	+	+	+

+ Gene product present.
− Gene product absent.

in the *L. plantarum* strains. Table 5 summarizes the presence and absence of plantaricin genes in the strains of this study. The plnA gene was found in all strains.The presence of the plnA gene suggests that they belong to plantaritype group 1 and group 2, which share the common feature of the plnABC regulatory system (Rizzello et al. 2014). The plnABC and plnEF were only found in strains S14, S31, and S34, respectively, the plnF found in strains S34 and U10, and the sequence of region plantaricin W gene in class lantibiotic were found in three of the strains (S31, S34, and U10). Plantaricin W are relatively rare among bacteriocinogenic *L. plantarum* strains (Holo et al. 2001). Although highly conserved, the genes plnJK was variously detected in the *L. plantarum* strains S31, S34, T3, T8, and U10. These results are in agreement with other studies that have reported these genes as the most prevalent (Omar et al. 2008).

In an effort to identify the isolates at the species level, molecular phylogeny analysis was carried out and a phylogenetic tree was constructed based on the 16s rDNA sequence from evolutionary distance using the neighbor-joining method. Although the 16s rDNA sequence analysis method is very good at identifying the organism by genus and species, it cannot differentiate strains at the subspecies level and is therefore it is not the appropriate method for measuring intraspecies relationships (Stackebrandt and Goebel 1994).

The isolates of our collection were identified based on 16s rDNA gene amplification and sequencing using the universal primer. Analyses of a fragment of approximately 1465 bp (position 27–1492 in *Escherechia coli*), comprising the hypervariable regions of *Lactobacillus*, allowed the construction of the phylogenetic tree, which is shown in Figure 1.

The phylogenetic (neighbour-joining) tree showed the relationship between *L. plantarum* isolate in this study and another *Lactobacillus* base on aligned 16s rDNA sequence. BLAST search analysis using the whole 16s rDNA sequence resulted in identification above the 99% level, although in two instances (U10 and S14) it was slightly lower. All homologies displayed were, however, above 97%, which is considered to be the cutoff value indicating species identity (Stackebrandt and Goebel 1994).

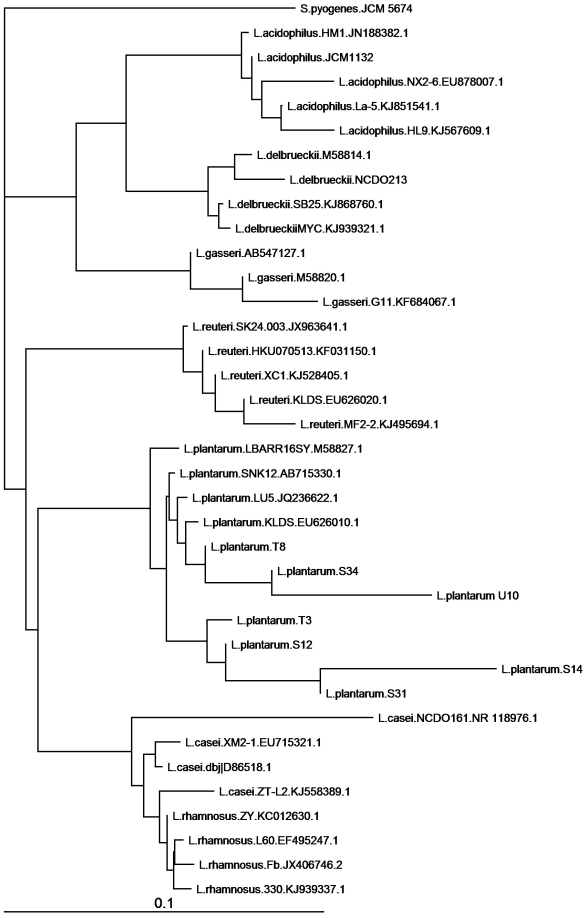

FIGURE 1 Phylogenetic tree showing the relative positions of lactic acid bacteria S12, S14, S31, S34, T3, T8, and U10 as inferred by the neighbor-joining method of complete 16s rDNA sequence. References of the type strains used for comparison are given, as well as the accession number for all 16s rDNA sequence. *Streptococcus pyogenes* is used as an outgroup. The horizontal branches are drawn proportionally to the number of nucleotide substitutions per site. The bar indicates 10% sequence divergence. *L.*, *Lactobacillus*; *S.*, *Streptococcus*.

4. Conclusions

In this study, the supernatant of *L. plantarum* strains under investigation showed a broad-spectrum of antimicrobial activity against pathogens. In fact, the activity appeared to have a heat-resistant characteristic and stable at a wide pH range. Besides, observed degradation of bacteriocins in the presence of proteolytic enzymes indicated its safety for being consumed by humans. Furthermore, almost all of *L. plantarum* strains under observation contained plantaricin-encoding genes, which were grouped into one cluster as indicated by phylogenetic analysis. Therefore, this study concluded that some of *L. plantarum* strains isolated in this study offered their potential to act as antimicrobial agents.

Acknowledgments

The authors are grateful to Laboratory for Applied Genetic Engineering and Protein Design, Research Center for Biotechnology-LIPI Cibinong.

Authors' contributions

AZM was designed this study. S carried out laboratory work and analyzed data. AZM and IMA advised about the laboratory technique and conducted manuscript proofreading before submission. All authors read and approved the final version of the manuscript.

Competing interests

The authors declare no other competing interests.

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