

## Original article

# Isolation and Identification of potentially bacteriocin-producing lactic acid bacteria from Sawi Asin

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### Abstract

The objective of this study are to isolate lactic acid bacteria from sawi asin and its antimicrobial activity in inhibiting the growth of *Staphylococcus aureus* and *Escherichia coli*. Screening of 66 bacterial isolates found 2 isolates of lactic acid bacteria that potentially produced bacteriocins. They were isolate PB3.6 and PG1.9, identified as *Lactobacillus plantarum* and *Lactobacillus brevis*, respectively. Isolates PB3.6 and PG1.9 could produced bacteriocins and able to inhibit the growth of *S. aureus* and *E. coli*. Bacteriocins of PB3.6 and PG1.9 isolates expected to be used as an alternative preservative agent in food products due to its capability of inhibiting the growth of food spoilage microbes, thus the usage of chemical additive material can be reduced.

## INTRODUCTION

Sawi asin or fermented mustard is an originally food product from Indonesia. It has been manufactured and consumed for many years in several regions of Indonesia. The mustard is fermented by adding salt and liquor from boiled rice to the vegetable. The fermentation process is indicated with the growth of several lactic acid bacteria, i.e. *Leuconostoc mesenteroides*, *Lactobacillus confusus*, *L. curvatus*, *L. plantarum*, and *Pediococcus pentosaceus* (Puspito & Fleet, 1985).

The fermented mustard is a great source to investigate lactic acid bacteria (LAB) (Chao et al., 2009). The LAB has a major role to play in preserving foods from spoilage microbes. Its role is linked to its ability to produce organic acids, peroxide hydrogen, diacetyl, anti-fungi, and bacteriocin compounds that are antagonistic against other microbes (Nespolo & Brandelli, 2010). Nowadays, numerous preservation methods have been used to avoid food spoilage. The

utilization of chemical preservative material is effective in preventing pathogens on food. Nevertheless, it is concerned that the residue that is left in human body can lead to serious disease in the future. The increasing cases of bacterial resistance of antibiotics, demand on save foods with only small amount of additive chemical materials, increases interests to replace the chemical additive compounds with safer organic materials. The solution proposed to address the issue is by using antimicrobial compounds such as bacteriocin that are produced by LAB.

Several LAB strains associated with food produce bacteriocin. Bacteriocin is a protein compound that exhibits bactericidal activity against closely related microbes (Nespolo & Brandelli, 2010). The compound is capable of inhibiting several bacteria that are responsible for damaging foods, such as *Listeria monocytogenes*, *Staphylococcus aureus*, and closely related bacteria (Parada et al., 2007). One of bacteriocins which is well-known and has been applied

as food preservative is nisin. National Agency of Drug and Food Control of the United States of America has granted Generally Recognized as Safe (GRAS) status for LAB and its nisin product (Gautam & Sharma, 2009). Nisin is proven capable to inhibit several Gram positive bacteria such as *Listeria*, *Clostridium*, *Bacillus*, and *Enterococcus*, but not effective to inhibit Gram negative bacteria, yeast, and mold.

Sulistiani et al., (2014) isolated several LAB from sawi asin of Central Java, Indonesia. Among others are *Lactobacillus farciminis*, *L. fermentum*, *L. namurensis*, *L. plantarum*, *L. helveticus*, *L. brevis*, *L. versmoldensis*, *L. casei*, *L. rhamnosus*, *L. fabifermentans*, and *L. satsumensis*. However, study related to bacteriocin produced by LAB isolated from sawi asin has not carried out yet. Therefore, the present study aims to isolate LAB from fermented mustard and to characterize its bacteriocin in inhibiting the growth of *Staphylococcus aureus* and *Escherichia coli*.

## MATERIALS AND METHODS

A total of 1 g sawi asin was mashed and put into 9 mL NaCl 0.85% prior to 10<sup>-1</sup>-10<sup>-6</sup> serial dilutions, and then it was inoculated on Man Rogosa Sharpe Agar (MRSA) containing 0.5% CaCO<sub>3</sub> (Desniar et al., 2013), incubation were conducted in anaerobic jar at room temperature for 48 hours. Isolates with clear zone on the media were then purified and inoculated onto slant MRSA media as stocks.

Antimicrobial activity of LAB was tested using bacterial culture followed by testing using selected supernatant isolates. The pH of the supernatant was neutralized using 1 M NaOH (pH 7.0 ± 0.2). Antimicrobial activity of LAB against *S. aureus* and *E. coli* was tested using disc diffusion method. A total of 80 µL bacterial culture/supernatant was put onto paper disc. One mL tested bacteria were inoculated into 100 mL Nutrient Agar (NA) media containing 0.8% agar. The inoculated bacteria in the media were then poured onto petri dishes and allowed to become solid. The paper disc was then put on the surface of the media prior to incubation at 37°C for 24 hours. The antimicrobial activity of LAB was determined from the formation of inhibition (clear) zone. The diameter of inhibition zone was calculated using the following equation below:

$$\frac{\text{diameter of inhibition zone (mm)} - \text{diameter of paper disc (mm)}}{\text{diameter of paper disc (mm)}}$$

Selected bacterial genomic DNA was extracted using Genomic DNA Mini Kit (Blood/Cultured Cell) (Geneaid GB100). Amplification of 16S rRNA gene was

performed by PCR using specific primers 63f (5'-CAG GCC TAA CAC ATG CAA GTC-3') and 1387r (5'-GGG CGG WGT GTA CAA GGC-3') (Marchesi et al., 1998). The PCR reaction was performed in a total volume of 50 µL containing 25 µL Go Taq Green Master Mix 2X, 2 µL each primer, 4 µL DNA template, and 17 µL nuclease free water.

The PCR was performed under the following conditions: pre-denaturation at 94°C for 4 minutes, denaturation at 94°C for 30 seconds, annealing at 55°C for 30 seconds, elongation at 72°C 1 minute with 30 cycles, post-elongation at 72°C for 7 minutes, and cooling at 4°C for 15 seconds. The PCR products were purified and sequenced according to standard from sequencing services company. Sequences were analyzed using MEGA 6 software then aligned with the 16S rRNA gene data base using BLAST-N program. Phylogenetic analysis was performed using MEGA 6 software with 1000x bootstrap.

## RESULT AND DISCUSSION

Isolation of LAB on MRSA media + 0.5% CaCO<sub>3</sub> from 6 sawi asin samples from Bogor resulted in 66 bacterial isolates (Table 1). The LAB bacteria were indicated by formation of clear zone around bacterial colonies (Figure 1). LAB isolates on MRSA + 0.5% CaCO<sub>3</sub> were indicated by the presence of clear zone around bacterial colonies. After 2-3 incubation days the LAB isolates produced acid that reacted with CaCO<sub>3</sub> and eventually clear zone was formed because Ca-lactate in the media was dissolved (Djide & Wahyudin, 2008). Fermented mustard is a suitable source for exploring lactic acid bacteria (LAB) (Chao et al., 2009). LAB held the main role to ferment sawi asin (Puspito & Fleet, 1985).

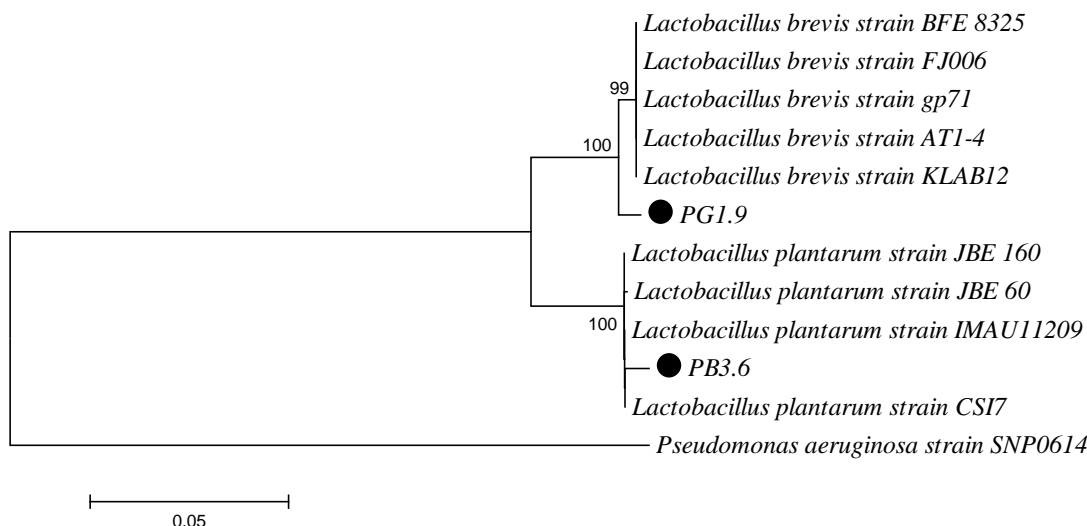


Fig 1. Clear zones of LAB isolates

A total of 66 purified LAB isolates were selected by their ability to inhibit *S. aureus* and *E. coli*. Ten isolates of LAB were selected based on the highest inhibitory index (Table 2). PB3.6 isolate had the highest inhibitory index against *S. aureus* and *E. coli* of 1.13 and 0.83, respectively. PB2.4 isolate showed the highest

inhibitory index against *E. coli* of 0.83. The activity of LAB shows that LAB produce antimicrobial compounds that can inhibit the growth of *S. aureus* and *E. coli*. The antimicrobe compounds produced by LAB are organic

acids, hydrogen peroxide, CO<sub>2</sub>, diacetylc, acetaldehyde, D-isomer amino acid, reuterin, and bacteriocin (Yang et al., 2012).



**Fig 2.** Phylogenetic tree of isolates PB3.6 and PG1.9 16S rRNA using neighbor joining method with 1000x bootstrap value

**Table 1.** Result of LAB isolation from sawi asin from Bogor

Sampling Location	Number of Samples	Sample Code	LAB Number
Anyar Market	2	PA1	16
		PA2	14
Bogor Market	3	PB1	12
		PB2	8
		PB3	6
Gunung Batu Market	1	PG1	10
Total	6		66

**Table 2.** Antimicrobial Activity of LAB Isolates

LAB Isolates	Inhibition Index			
	Cell Culture		Supernatant	
	<i>S. aureus</i>	<i>E. coli</i>	<i>S. aureus</i>	<i>E. coli</i>
PA2.1	0.92	0.38	0.13	0.31
PA2.13	0.60	0.60	0.13	0.25
PB1.4	0.54	0.67	0.25	0.25
PB1.11	0.63	0.63	0.17	0.29
PB1.12	0.60	0.71	0.35	0.31
PB2.2	0.50	0.75	0.38	0.31
PB2.4	0.50	0.83	0.31	0.33
PB3.4	0.50	0.79	0.31	0.31
PB3.6	1.13	0.83	0.50	0.40
PG1.9	0.75	0.25	0.42	0.50

Then the ten isolates were tested for their supernatant antimicrobial activity. The pH of the supernatant of isolates was neutralized using 1 M NaOH to eliminate the antimicrobial effect of organic acid produced by the bacteria. This is an initial test to estimate the ability of lactic acid bacteria isolates to produce bacteriocin. Two isolates with the highest inhibition index were isolates PB3.6 and PG1.9 (Table 2). Supernatant of isolates PB3.6 and PG1.9 inhibited the growth of *S. aureus* and *E. coli* and both were potentially produced bacteriocin. Bacteriocin is a protein compound that can inhibit the growth of closely related bacteria. Bacteriocin is synthesized in the ribosome and excreted extracellularly and has bactericidal or bacteriostatic activity (Jeevaratnam et al., 2005; Bali et al., 2011).

Isolates PB3.6 and PG1.9 with the highest inhibition index of supernatant against *S. aureus* and *E. coli* were selected for further identification. Isolates PB3.6 and PG1.9 were bacilli Gram positive bacteria. Identification of isolate were carried out using 16S rRNA genes. Analysis on the sequence of 16S rRNA gene and data in Gene Bank using BLAST-N program showed that isolate PB3.6 was 96% similar to *Lactobacillus plantarum* strain JBE 160, while isolate PG1.9 was 98% similar to *Lactobacillus brevis* strain KLAB12. Analysis on phylogenetic tree also showed that isolate PB3.6 was highly related to *Lactobacillus plantarum* and isolate PG1.9 to *Lactobacillus brevis* (Figure 2). Various studies reported on the capability of *L. plantarum* and *L. brevis* in producing bacteriocin, where *L. plantarum* produces plantaricin and *L. brevis* produces brevisin (Coventry et al., 1996; Wen et al., 2016).

## CONCLUSION

Isolation of lactic acid bacteria from *sawi asin* resulted 10 isolates that inhibited the growth of *S. aureus* and *E. coli*. Supernatant of isolates PB3.6 and PG1.9 had the highest inhibition index and potentially produced bacteriocins. Isolates PB3.6 and PG1.9 identified as *Lactobacillus plantarum* and *Lactobacillus brevis*, respectively.

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