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Production and Application of *Lactobacillus plantarum* IBL-2 Bacteriocins as Meat Product Biopreservatives

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Abstract

Bacteriocin produced by Lactic Acid Bacteria (LAB) is a potential candidate for biopreservatives, which is safe for consumption, since it can be degraded by proteolytic enzymes. The aim of this study was to optimize bacteriocin production from *Lactobacillus plantarum* IBL-2 and to evaluate the effectiveness of bacteriocins in decreasing the total number of several tested bacteria using total plate count method and *Salmonella typhimurium* spiked in ground beef. Bacteriocin was produced through fermentation of *L. plantarum* IBL-2, under various conditions to yield the compound with the best antibacterial activity. The total number of bacteria in ground beef after the addition of *L. plantarum* IBL-2 fermentation supernatant was determined. The result was compared with the sample without preservatives (control), and sample added with commercial Nissin. The fermentation process resulted in bacteriocin with the strongest antimicrobial activity when using low molecular weight liquid medium (LMWLM), followed by a series of refining process. The highest antibacterial activity of bacteriocin was obtained using LMWLM as fermentation media, and using a series of refining process consist of bacteriocin supernatant evaporation, membrane ultrafiltration, and gradual fractionation using 80% ammonium sulphate. Bacteriocin from *L. plantarum* IBL-2 showed antimicrobial activity against *S. typhimurium*.

Keywords: bacteriocin production, *Lactobacillus plantarum* IBL-2, biopreservative, meat product biopreservative

Introduction

Assurance of food quality and safety is vital for consumers' health. One of the quality and safety issues is spoilage caused by microorganism, which is prevented by adding preservatives. In recent years, nitrate and sodium nitrite as a chemical preservatives have been widely used in meat and dairy processing with a suitable antimicrobial effect. However, it has been reported that application of sodium nitrite in a high amount in canned fish meal for domestic animals fed

in 1960 triggered liver toxicity of the animals (Kashani et al., 2012).

Currently, lactic acid bacteria (LAB) and antimicrobial compound synthesized by LAB are considered to be natural preservatives or biopreservatives. The preservation substance produced by LAB has been identified as peptides which are generally known as 'bacteriocins' (Udhayashree et al., 2012; Jeevaratnam et al., 2005). Bacteriocin is a protein substance which has bactericidal and bacteriostatic activity (Kemperman et al.,

2003). Bacteriocins are generally named based on the genus or species of the strain that produce the bacteriosin. For instance, *Lactobacillus plantarum* produce plantaricin (Yusuf, 2013). Nowadays, bacteriocins have been widely used especially in the field of food preservation. The use of bacteriocins in food industry especially on dairy, egg, vegetable and meat products has been extensively investigated. Bacteriocins can be applied on purified, semi-purified or on a crude form. Besides, it can be applied through the use of a product that has been previously fermented with a bacteriocins producing strain or through the directly use of bacteriocin producing strain (Siamansouri et al., 2013; Karapetyan et al., 2010).

L. plantarum IBL-2 (LP IBL-2) is one of the LABs obtained from Biotechnology Culture Collection (BTCC) LIPI Cibinong which has the highest activity against several microorganism (Nurhayati et al., 2013). However, the potency for bacteriosin producer has not yet been studied. This research was aimed to optimize bacteriocin production from *L. plantarum* IBL-2 and to evaluate the effectiveness of bacteriocins in reducing the number of Total Plate Count (TPC) and *Salmonella typhimurium* in ground beef

Materials and Methods

Microorganisms

Lactobacillus plantarum IBL-2 or LP IBL-2 was used as a bacteriocin producing strain. The bacteria were obtained from Biotechnology Culture Collection (BTCC) LIPI Cibinong. The bacteriocin was obtained from the supernatant of fermentation using LP IBL-2

Food-borne disease bacteria including *Staphylococcus aureus* ATCC 25923, *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, *Salmonella typhimurium* ATCC 14028 and *Listeria monocytogenes* (BTCC) were used to test the

bactericidal and bacteriostatic activity of the bacteriocin produced by LP IBL-2

Food Product

Ground beef was used to evaluate the effectiveness of bacteriosin in food product. The ground beef was obtained commercially.

Chemicals

A commercial bacteriosin and nisin were used to compare the performance of bacteriocin produced by LP IBL-2. Nisin was used at concentration of 2.5% (w/v). It was purchased from Sigma Chemical CO., USA.

Optimization of LP IBL-2 Production

Several growth media including, De Man-Rogosa-Sharpe broth (MRSB), modified nutrient broth (NB), Low molecular weight liquid medium (LMWLM) (Zacharof and Lovitt, 2012), and Tryptone Glucose Yeast (TGY)-Extract were tested in order to obtain the highest LP IBL-2 production. LP IBL-2 in glycerol 20% was inoculated in MRSB medium for two times. Subsequently, 1-2% LP IBL-2 was inoculated in 100 mL of different tested media with pH of 6-7. The media were then incubated at 30°C for 24h. After the incubation, the culture was centrifuged at 20000 g for 15 min at 4°C (Todorov and Dicks, 2004). The supernatant was heated at 80°C for 10 min to prevent bacteriocin proteolysis (Todorov and Dicks, 2004). Supernatant was then neutralized by adding NaOH 2 M, followed by filtration using 0.2 µm kDa MWCO filter. Antimicrobial activity of each supernatant from different media was tested on several bacteria (CLSI, 2009). Medium that yields the highest antibacterial activity of LP IBL-2 was used further in the purification of bacteriosin.

Purification of bacteriocin supernatant LP IBL-2

LP IBL-2 (10%) was inoculated into 2000 mL fermenter containing medium selected from the previous work at pH of 6-7. It was then incubated at 30°C for 24 h. The fermentation was carried out in batch method. At the end of fermentation, the broth was centrifuged at 4°C using 20000 g for 15 min. The supernatant was heated at 80°C for 10 min to prevent bacteriocin proteolysis (Todorov and Dicks, 2004) followed by neutralization using NaOH 2 M. To purify the bacteriocin supernatant of LP IBL-2, the supernatant was concentrated using evaporation method, followed by gradual ultrafiltration using several membranes (40 KD MWCO, 10 KD MWCO and 1 KD MWCO). The retentate obtained from ultrafiltration using 1KD MWCO was gradually fractionated with ammonium sulfate (Scopes, 1989) of The Bacteriocins of LP IBL-2 obtained from each step of purification was tested to evaluate their antibacterial activity (CLSI, 2009).

Application of bacteriocin on ground beef

Minimum Inhibitory Concentration (MIC) for both purified bacteriocins supernatant LP IBL-2 and nisin was first determined using agar diffusion method (CLSI, 2009) prior to application on ground beef.

Effectiveness of the bacteriocin was evaluated both on fresh and cooked ground beef spiked with *S.typhimurium*. Concentration of both purified bacteriocins supernatant LP IBL-2 and nisin used in this test was five times of their MIC. Cooked ground beef was prepared by sterilization of

ground beef in autoclave at 121°C for 15 min followed by inoculation of 10⁵ CFU/ml of *S.typhimurium*. One milliliter of purified bacteriocin supernatant LP IBL-2 and nisin were added to 20 g of each fresh and cooked ground beef spiked with *S.typhimurium*. Twenty grams of fresh and cooked ground beef spiked with *S.typhimurium* without addition of any substance were used as a control. The ground beefs were then stored in refrigerator at 4-10°C. The bacterial growth was determined on day 0, 2, 6, 8, 12, and 14 using TPC method. For measurement the growth of *S.typhimurium* on cooked ground beef, the TPC method was performed in a selective media XLDA.

Statistical analysis

The data for antibacterial activity of bacteriocin supernatant LP IBL-2 presented are the average from triplicate. To determine significance difference of each treatment on ground beef, the TPC result and number of *S.typhimurium* were analyzed using ANOVA.

Results and Discussion

The main goal of the optimization process was to obtain growth medium which yield bacteriocin with the highest antibacterial activity. The type of medium and its' composition is presented in **Table 1**.

The media for optimization were selected through literature review and also through the consideration of production cost. The antibacterial activity of bacteriocin yielded from each medium is presented in **Table 2**.

Table 1. The Type and Composition of Medium for Bacteriocin Production from LP IBL-2

Medium Composition	Type of Medium			
	MRSB (g/L)	Modified NB (g/L)	LMWLM (g/L)	TGY-Extract (g/L)
Glucose	20.0	20.0	20.0	10.0

Yeast Extract	4.0	2.0	2.0	3.0
Meat Extract	8.0	-	-	-
Peptone	10.0	5.0	-	-
Tryptone	-	-	-	5.0
K ₂ HPO ₄	2.0	-	0.5	-
Sodium acetate trihydrate	5.0	-	1.0	-
Triammonium citrate	2.0	-	1.0	-
Lab Lemco Powder	-	1.0	-	-
NaCl	-	5.0	-	-
MgSO ₄ 7H ₂ O	0.2	-	0.2	-
MnSO ₄	0.05	-	0.05	-

As presented in **Table 2** only Modified NB that was significantly different with other media. Hence, MRSB, LMWLM and TGY-Extract are equally good for the production of bacteriocins LP IBL 2. However, the highest inhibitory activity was obtained from the bacteria grown in LWLM. This result is in accordance with a previous study reporting that LMWLM medium enhanced the growth of *Lactobacillus* and enhanced bacteriocin production (Zacharof and Lovitt, 2012). The use of LWLM was also preferred due to its' feature which allows filtration and filtration is an efficient method to separate bacteriocin from other protein produced during fermentation process.

Production Process of bacteriocin LP IBL-2

One of the challenges in bacteriocin productions was the low yield of bacteriocin from fermentation broth, therefore concentrating and refining steps play an important role in the production process (Pingitore et al., 2007). As stated in the material and method, optimum production step consisted of fermentation, centrifugation, obtaining supernatant, neutralization of supernatant, heating, evaporation, followed by a series of ultrafiltration and precipitation. Evaporation was conducted to minimize the volume of water which eventually would ease the filtration process. Ultrafiltration method was selected since it allows concentrating process

Table 2. Inhibitory Diameter of Bacteriocins Yielded from different Media

Type of Medium	Inhibitory diameter (mm) of bacteriocin supernatant LP IBL-2 60% (v/v) from various media against various bacteria					
	<i>S. aureus</i>	<i>E. coli</i>	<i>P. aeruginosa</i>	<i>S. typhi</i>	<i>L. monocytogenes</i>	Average
MRSB	10.0	10.0	11.0	11.0	11.2	10.6
Modified NB(*)	9.0	9.0	8.0	8.0	8.0	8.4
LMWLM	11.3	10.1	11.2	11.9	11.2	11.1
TGY-Extract	8.0	8.5	9.8	9.8	12.0	9.6

*Significance difference compare to MRSB and LMWLM (p<0.05)

Table 3. Inhibitory Diameter of Supernatant LP IBL-2 after Each Purification step

Bacteria	Crude bacteriocin	Evaporated bacteriocin	Filtrated bacteriocin (R3)	Precipitated bacteriocin
<i>S. aureus</i>	11.3	12.0	13.0	14.0

<i>E. coli</i>	10.1	13.0	13.5	15.0
<i>P. aeruginosa</i>	11.2	12.0	14.0	14.0
<i>S. typhi</i>	11.9	12.8	14.0	16.5
<i>L. monocytogenes</i>	11.2	13.0	14.0	14.0
Mean	11.1	12.4	13.7	14.7

along with refining through the separation of protein molecule based on its' molecular weight. During ultrafiltration process using several membranes with different pore diameter, three retentates were obtained *i.e.*, retentate 1 (R1), retentate 2 (R2), and retentate 3 (R3) which may contain protein with molecular weight of ≥ 40 KD, 10 – 40 KD, and 1 -10 KD, respectively. Based on theoretical molecular weight of bacteriocins and activity testing, it was concluded that bacteriocins was obtained in R3. The inhibitory diameter of the result of each process is presented in **Table 3**.

Table 3 shows that there was an increase of antibacterial activity after each purification steps against various bacteria. Precipitated bactericin gave 1.3 times higher activity than that of the crude bacteriocin. The increase was due to the filtration and precipitation process which resulted in higher concentration hence it showed stronger antibacterial activity. **Table 4** shows antibacterial activity against *S. typhimurium* and the yield of each purification steps.

Similar to the previous result, antibacterial activity against *S. typhimurium*

also increase after each step of purification. The increase of antibacterial activity was followed with the decrease in the volume. The yield of the bacteriocin at the end of the purification step was 7.7% of the crude bacteriocin. The similar research of bacteriocin production process has been carried out and patented by European Patent application with publication number of 0445414 A2 (1991), in which the yield of bacteriocin reached 23%, while the bacteriocin yield obtained in other work was only 2% (Stauber and Scherer, 1994)

Determination of minimum inhibitory concentration (MIC) of purified bacteriocin supernatant LP IBL-2 and Nisin

Agar diffusion method was used to determine the MIC. The result showed that the MICs of purified bacteriocin supernatant LP IBL-2 and nisin were , 0.08% (w/v) and 2.88% (v/v), respectively. Hence, it can be concluded that 2.88% (v/v) of bacteriocin supernatant LP IBL-2 was equal to 0.08% (w/v) of nisin.

Table 4. Antibacterial activity against *S. typhimurium* and Yield of Bacteriocin Supernatant LP IBL-in each step of purification

Type of bacteriocin	Volume (mL)	Activity (mm ² /mL)	Total Activity (mm ²)	Yield* (%)
Crude	2000	5281	1,06x10 ⁷	100
Evaporated	1000	6392	6,39x10 ⁶	60,3
Filtrated (R3)	180	8000	1,44x10 ⁶	13,6
Precipitated	60	13613	8,17x10 ⁵	7,7

*Yield (%) is calculated as total activity of subsequent step x100/ Total activity of crude preparation (Jabeen et al., 2009).

Application of purified bacteriocin supernatant LP IBL-2 on fresh ground beef

The inhibitory activity of purified bacteriocin supernatant LP IBL-2 was assayed in fresh ground beef. The result was compared

with commercial nisin and fresh ground beef without addition of any antibacterial compound was used as control. The concentration of purified bacteriocin supernatant LP IBL-2 and nisin added to the

ground beef was five times higher than their MIC, since it was predicted that effective antibacterial inhibition would be obtained at this concentration. The concentrations of purified bacteriocin supernatant LP IBL-2 and nisin used in this work were 0.4% (w/v) and 14.4% (v/v), respectively. The TPC of each fresh ground beef during 14 days storage was presented in **Table 5**.

Table 5 showed that on day 0, prior to the addition of biopreservatives, ground beef added by bacteriocins supernatant LP IBL-2 or nisin contained lower number of bacteria. This indicated that both biopreservatives possessed inhibitory action on initial addition. According to statistical analysis, it was found that the addition of nisin and bacteriocin supernatant LP IBL-2 influenced the bacterial growth in fresh ground beef, since the TPC for both of them showed significance difference compared to control ($p < 0.05$). The reduction of viable bacteria in ground beef added by bacteriocin supernatant LP IBL-2 was greater compared to nisin, which is due to the fact food spoilage bacteria were mostly gram negative bacteria and bacteriocin was sensitive against the type of bacteria. According to Indonesia's National Standard (SNI 3932:2008), the maximum limit of TPC

for meat product is 1×10^6 CFU/g; therefore the bacteria growth for each treatment was below the standard. This may be caused by double preservation method which is through the addition of biopreservatives (Ananou et al., 2007) and storage in cold temperature (Fardiaz, 1992).

Application of bacteriocin on cooked ground beef spiked with *S. typhimurium*

Food safety is not just determined through the total number of viable bacteria, but also the type of pathogen bacteria contained in the food product. One of several dangerous food contaminant bacteria is *S. typhimurium* since it may cause typhoid fever. Ground beef is a suitable medium for *S. typhimurium* growth due to the water content and several important nutrients contained in ground beef, as well as the pH which support the bacterial growth. Therefore, high quality meat product can be obtained through minimizing the number of viable *S. typhimurium*. **Table 6** presents the result of TPC of each cooked ground beef spiked with *S. typhimurium* with addition of bacteriocin LP IBL-2 and nisin or without addition of any biopreservative (control) during 14 days storage.

Table 5. Total Plate Count for Fresh Ground Beef

No	Treatment	TPC (Day of Storage)					
		0	2	6	8	12	14
1.	Nisin (*) Purified	5.57±0,00	4.00±0,0	5.24±0,00	5.47±0,01	5.44±0,03	5.37±0,00
2.	bacteriocins supernatant LP IBL- 2 (*)	5.68±0,00	5.04±0,00	5.08±0,18	5.07±0,01	4.96±0,02	4.62±0,11
3.	Control	5.70±0,00	5.36±0,00	5.62±0,01	5.65±0,06	5.60±0,12	5.56±0,03

*Significance difference compare to control ($p < 0.05$)

Table 6. Total *S. typhimurium* on Cooked Ground Beef

No	Treatment	Total <i>S. typhimurium</i> (\log_{10} CFU/g) on Certain Day of Storage					
		0	2	6	8	12	14
1.	Nisin (*)	3.25±0,00	3.23±0,00	3.00±0,00	2.95±0,00	2.70±0,10	2.90±0,00

	Bacteriocins supernatant LP IBL-2 (*)						
2.	Bacteriocins supernatant LP IBL-2 (*)	3.38±0,00	3.36±0,08	2.42±0,35	2.23±0,18	2,00±0,06	1.95±0,14
3.	Control	4.48±0,00	4.23±0,11	4.21±0,01	4.21±0,01	4.18±0,01	4.50±0,03

*Significance difference compare to control ($p < 0.05$)

For ground beef added with bacteriocin supernatant LP IBL-2 and Nisin, a decrease in *S. typhimurium* number on day-0 prior to the addition of biopreservatives revealed that both biopreservatives was able to inhibit *S. typhimurium* growth upon initial addition. Bacteriocin supernatant LP IBL-2 was able to decrease the number of viable bacteria less than 0.1% from day 0 to day 14. Gram negative bacteria such as *S. typhimurium*, has layered cell wall, in which the polysaccharide structure of the cell wall built a system where only selected substance may enter (Davidson and Braner, 1983). Due to the fact, biopreservatives usually took some times to penetrate the bacteria and inhibit the growth. Some researchers reported that bovine meat cubes dipped in the filtered and neutralized supernatant of the fermented broth presented lower counts than those recommended by ICMSF as quality standards for raw meat ($<10^7$ CFU/g) for the sixth day (Fiorentini et al., 2001). The other researchers reported that bacteriocin from *L. plantarum* 2C12 could inhibit pathogenic bacteria such as *E. coli*, *S. aureus* and *S. typhimurium*. The bacteriocin concentration of 0.3% has been reported to pose similar effectivity as nitrate as biopreservative of meat balls by inhibiting the growth of total microbes and *E. coli* on the sixth day (Arief et al., 2012).

Conclusion

LMWLM is the most suitable medium to produce bacteriocin from LP IBL-2 since it gave the highest antibacterial activity. Purification processes including a series of

ultrafiltration and precipitation increased the antibacterial activity at the same time decreased the volume of the supernatant. The bacteriocin yield after purification was 7.7%. The antibacterial activities against several tested bacteria and *S. typhimurium* were 1.3 and 1.39 times higher than that of their crudes, respectively. The bacteriocin supernatant LP IBL-2 was able to inhibit the growth of $1.04 \log_{10}$ CFU/g total bacteria in ground beef and $1.43 \log_{10}$ CFU/g of *S. typhimurium* in cooked ground beef for 14 days.

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