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4501 Words

CHARACTER COUNT

24440 Characters

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8 Pages

FILE SIZE

1.1MB

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Feb 6, 2023 8:20 AM GMT+7

REPORT DATE

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The Effect of Carbon and Nitrogen Supplementation on Bacteriocin Production of Lactic Acid Bacteria from Pickled Yellow Bamboo Shoots (*Dendrocalamus Asper*)

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1 Six selected lactic acid bacteria (LAB) isolates from pickled Yellow Betung bamboo shoots were grown in the Mann Rogosa Sharpe-Broth (MRSB) media with different supplementation combination. The cell-free supernatant were evaluated for their ability to produce bacteriocin by adjusting its pH to 6.0 in order to remove organic acid effects. The bacteriocin activity was assayed by agar-well diffusion method. The inhibitory activity calculated in Activity Unit (AU in $\text{mm}^2 \text{mL}^{-1}$) of bacteriocins. The aims of this paper is to explore the effect of different medium compositions on bacteriocin production and its inhibitory activity against pathogenic bacteria (*Listeria monocytogenes* FNCC 0156, *Staphylococcus aureus* FNCC 0047, and *Escherichia coli* FNCC 0091). Supplementations of carbon and nitrogen sources induced production of bacteriocins. LAB isolates grown in media without supplementation could not produce bacteriocins. Growth of isolate D44 in the presence of 2% of glucose and 2% of yeast extract yielded the largest bacteriocin inhibitory activity levels of 3179 AU mL^{-1} against *Listeria monocytogenes* FNCC 0156, 4663 AU mL^{-1} against *Staphylococcus aureus* FNCC 0047, and 3109 AU mL^{-1} against *Escherichia coli* FNCC 0091.

Key words: bacteriocin, lactic acid bacteria, pickled bamboo shoots, supplementation

2 Enam isolat bakteri asam laktat terpilih (LAB) yang diisolasi dari acar rebung bambu kuning ditumbuhkan di media Mann Rogosa Sharpe-Broth (MRSB) dengan kombinasi suplementasi yang berbeda. Supernatant bebas sel diatur pH menjadi 6,0 untuk menghilangkan efek asam organik dan dievaluasi aktivitas bakteriosinnya. 19 Aktivitas bakteriosin diuji dengan metode difusi agar-well. Aktivitas penghambatan dihitung dalam Unit Aktivitas (AU dalam $\text{mm}^2 \text{mL}^{-1}$) bakteriosin. Tujuan dari penelitian ini adalah untuk mengeksplorasi pengaruh komposisi medium yang berbeda pada produksi bakteriosin dan aktivitas penghambatannya terhadap bakteri patogen (*Listeria monocytogenes* FNCC 0156, *Staphylococcus aureus* FNCC 0047, dan *Escherichia coli* FNCC 0091). 2 Suplementasi sumber karbon dan nitrogen menginduksi produksi bakteriosin. Isolat LAB yang tumbuh di media tanpa suplementasi tidak dapat menghasilkan bakteriosin. Pertumbuhan isolat D44 dengan adanya 2% glukosa dan 2% yeast extract menghasilkan aktivitas penghambatan bakteriosin terbesar, yaitu 3179 AU mL^{-1} terhadap *Listeria monocytogenes* FNCC 0156, 4663 AU mL^{-1} terhadap *Staphylococcus aureus* FNCC 0047, dan 3109 AU mL^{-1} terhadap *Escherichia coli* FNCC 0091.

Kata kunci: acar rebung, bakteriosin, bakteri asam laktat, suplementasi

Bamboo shoots can be processed into fermented product such as vegetable pickle using lactic acid bacteria (LAB). LAB from different salt concentrations and temperatures have been isolated. Isolates were prepared from each fermentation conditions: fermentation in 2.5% salt solution at 15 °C (21 isolates), 5.0% salt solution at 15 °C (22 isolates), 2.5% salt solution at 30 °C (22 isolates), and 5.0% salt solution at 30 °C (27 isolates). All combinations of salt concentration and fermentation temperature yielded isolates with probiotic capability and antimicrobial activity (Lindayani *et al.* 2015).

Some lactic acid bacteria can produce bacteriocin (Salminen *et al.* 2004). Many studies conducted on bacteriocins found that the production of bacteriocin is

often regulated by some factors, such as growth pH, temperature, and nutrient sources (Mahrous *et al.* 2013; Todorov and Dicks, 2005; Zhou *et al.* 2014). Bacteriocin production requires suitable culture medium containing all essential nutrients in suitable amounts. Growth medium compositions, especially the sources and concentrations of carbon and nitrogen strongly affect production of bacteriocins (Khay *et al.* 2013; Todorov and Dicks (2005).

The effect of medium compositions on the bacteriocin inhibitory activity of LAB isolated from fermented bamboo shoot pickle is still unknown. The aims of this paper are to to compare the effect of different medium compositions on bacteriocin production and its inhibitory activity against pathogenic bacteria (*Listeria monocytogenes* FNCC 0156, *Staphylococcus aureus* FNCC 0047, and *Escherichia coli* FNCC 0091).

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MATERIALS AND METHODS

Bacterial Strains and Culture Conditions.

Eleven lactic acid bacteria (LAB) strains isolated from pickled yellow bamboo shoots (Lindayani *et al.* 2015; Hartayanie *et al.* 2016) were used in this study. The LAB were propagated in MRSB (Merck, Germany) containing 0.2% glucose (Merck, Germany) and incubated at 37 °C for 24 h. The microorganisms were propagated and maintained in NB (Nutrient Broth) medium. Three indicator microorganisms used in antibacterial activity tests were *Listeria monocytogenes* FNCC 0156, *Staphylococcus aureus* FNCC 0047, and *Escherichia coli* FNCC 0091. The indicator microorganisms were propagated in Nutrient Broth (Merck, Germany) at 37 °C for 24 h.

Antibacterial Activity. Antibacterial activity was tested by using the well diffusion method (Ivanova *et al.* 2000). Ten µL of pathogen inocula equivalent to McFarland 3 was inoculated with 10 mL of nutrient agar (NA, Merck, Germany) as a pour plate and was allowed to solidify. Twenty µL of cell-free supernatant was inoculated in 5.5 mm diameter wells and incubated for 3 h at 4 °C to let it be absorbed. Sterile MRSB was used as a negative control. Antibacterial activity was tested against pathogenic bacteria (*Staphylococcus aureus* FNCC 0047 and *Escherichia coli* FNCC 0091). Clear zone around the wells was measured after 24 h incubation at 37 °C.

Antibacterial activity was expressed as arbitrary units (AU) per mL. One AU was defined as the reciprocal of the highest dilution showing a clear zone of growth inhibition. The calculation of the antibacterial activity was the same as the bacteriocin activity calculation.

Identification of Bacteriocin-Producing Species. The LAB was identified based on its physiological and biochemical characteristic using API 50 CHL test strips (Biomereux, Marcy-l'Étoile, France). The identified isolates were kept at -18 °C in MRS broth containing 15% of glycerol (Merck, Germany).

Bacteriocin Bioassay. Bacteriocin activity testing was performed by using the well diffusion method (Ivanova *et al.* 2000). Ten µL of pathogen inocula equivalent to McFarland 3 was inoculated into 10 mL of nutrient agar (NA, Merck, Germany) as a pour plate and was allowed to solidify. Cell-free supernatant was neutralized by adjusting pH to 6.0 with 1 M NaOH in order to prevent the inhibitory effect of lactic acid (Karthikeyan and Santosh 2009). Twenty µL of

neutralized cell-free supernatant was inoculated in 5.5 mm diameter wells and incubated for 3 h at 4 °C to let it absorbed. Sterile MRSB was used as a negative control. Bacteriocin activity was tested using pathogenic bacteria (*Listeria monocytogenes* FNCC 0156, *Staphylococcus aureus* FNCC 0047 and *Escherichia coli* FNCC 0091). The clear zone around the wells was measured after 24 hours incubation at 37 °C.

Bacteriocin activity was expressed as arbitrary units (AU) per mL. One AU was defined as the reciprocal of the highest dilution showing a clear zone of growth inhibition.

$$\text{Bacteriocin activity} \left(\frac{\text{mm}^2}{\text{mL}} \right) = \frac{Az - As}{V}$$

Key:

Az : Clear zone area (mm²)

As : Well area (mm²)

V : Sample volume (mL)

Effect of Medium Composition on Bacteriocin

Production. The LAB isolates that have bacteriocin activity against pathogenic bacteria (*Listeria monocytogenes* FNCC 0156, *Staphylococcus aureus* FNCC 0047 and *Escherichia coli* FNCC 0091) were grown in MRSB at 37 °C for 48 h. The isolates adjusted to McFarland 5. Five hundreds µL of isolate was mixed with 500 µL of the following media: (i) MRSB without supplementation; (ii) MRSB supplemented with 2% glucose; (iii) MRSB supplemented with 2% glucose and 2% tryptone; (iv) MRSB supplemented with 2% glucose and 2% yeast extract; and (v) MRSB supplemented with 2% glucose, 1% tryptone, and 1% yeast extract. Then isolates were incubated in an incubator at 37 °C for 24 h. After incubation, the cells were separated by centrifugation at 11000 g for 10 min at 4 °C. After centrifugation the supernatant was collected in a fresh sterile tube and the pellet was discarded. The cell free supernatant (CFS) was neutralized by adjusting pH to 6.0 using 1 N NaOH for bacteriocin assays against three pathogenic bacteria (*Listeria monocytogenes* FNCC 0156, *Staphylococcus aureus* FNCC 0047 and *Escherichia coli* FNCC 0091).

RESULTS

All isolates that primarily confirmed as LAB from pickled Yellow Betung bamboo shoots were tested for antibacterial activity. The isolates showed antibacterial activity against *Listeria monocytogenes* FNCC 0156, *Staphylococcus aureus* FNCC 0047, and *Escherichia coli* FNCC 0091 (Table 1).

Different treatment in salt concentration and

temperature of fermentation gave various LAB (Table 2). However, *Leuconostoc mesenteroides* ssp. *cremoris* was found from fermentation condition A (A43) and C (C18 and C29). *Lactobacillus pentosus* was found from fermentation condition B (B1) and D (D44). Isolates with significant values more than 80% showed very good identification results (A20, B1, B31, B32, C19, D11, D20, and D44). The results of API kit identification of A43, C18, and C29 could not be accepted because the significant value was less than 80% (www.biomerieux-usa.com). Some rod-shaped isolates were acquired i.e., B1 as *Lactobacillus pentosus* (99.7%), C19 as *Lactobacillus plantarum* (99.6%) and D44 as *Lactobacillus pentosus* (96%). Cocci-shaped were found from isolate A20, B3, and B32. A20 was identified as *Leuconostoc mesenteroides* ssp. *mesenteroides/dextranicum 2* (96.8%), B31 was identified as *Enterococcus durans* (86.15%) and B32 was

identified as *Lactococcus lactis* ssp. *lactis 1* (96.5%).

Table 3 showed that there was no bacteriocin activity due to absence of supplementation. All isolates showed vary bacteriocin inhibitory activity against pathogen when they were grown in supplemented MRSB. C18 showed higher bacteriocin activity when it was cultured in MRSB supplemented with glucose and tryptone rather than in MRSB supplemented with glucose. Bacteriocin production of C19, C29, and D44 was improved by carbon and nitrogen supplementation. Tryptone was better supplement than yeast extract to improve bacteriocin production of C19. But on C29, yeast extract was better than tryptone. Bacteriocin production of D11 could only be induced by carbon supplementation.

Bacteriocin D44 could inhibit all pathogenic bacteria when produced in MRSB supplemented with combinations of carbon and nitrogen sources (tryptone

Table 1 Result of Antimicrobial Activity

Fermentation Condition	Isolate Code	Diameter of Inhibition Zone (mm)	
		<i>S. aureus</i> FNCC 0047	<i>E. coli</i> FNCC 0091
A	A20	9.70 ± 1.21	10.93 ± 2.47
	A43	10.70 ± 0.92	9.67 ± 1.81
B	B1	9.37 ± 1.00	9.50 ± 1.73
	B31	12.40 ± 0.00	9.87 ± 1.60
C	B32	12.70 ± 0.00	8.27 ± 1.10
	C18	14.70 ± 0.00	8.90 ± 0.00
D	C19	10.70 ± 0.00	11.90 ± 0.00
	C29	12.90 ± 0.00	9.80 ± 0.00
	D11	10.80 ± 0.00	10.80 ± 0.00
	D20	10.73 ± 2.06	7.10 ± 0.00
	D44	8.80 ± 0.00	10.90 ± 0.00

Key:

A = 2.5% of salt concentration at 15 °C
 B = 5.0% of salt concentration at 15 °C
 C = 2.5% of salt concentration at 30 °C
 D = 5.0% of salt concentration at 30 °C

Table 2 Result of API software identification (24 hours incubation)

Fermentation Condition	Isolate Code	Species Identification	Significant level (%)
A	A20	<i>Leuconostoc mesenteroides</i> ssp. <i>mesenteroides/dextranicum 2</i>	10.93 ± 2.47
	A43*	<i>Leuconostoc mesenteroides</i> ssp. <i>cremoris</i>	9.67 ± 1.81
B	B1	<i>Lactobacillus pentosus</i>	9.50 ± 1.73
	B31	<i>Enterococcus durans</i>	9.87 ± 1.60
	B32	<i>Lactococcus lactis</i> ssp. <i>lactis 1</i>	8.27 ± 1.10
C	C18*	<i>Leuconostoc mesenteroides</i> ssp. <i>cremoris</i>	8.90 ± 0.00
	C19	<i>Lactobacillus plantarum 1</i>	11.90 ± 0.00
	C29*	<i>Leuconostoc mesenteroides</i> ssp. <i>cremoris</i>	9.80 ± 0.00
D	D11	<i>Lactobacillus fermentum 1</i>	10.80 ± 0.00
	D20	<i>Lactobacillus s plantarum</i>	7.10 ± 0.00
	D44	<i>Lactobacillus pentosus</i>	10.90 ± 0.00

Key:

* = Significant < 80 %
 A = 2.5% of salt concentration at 15 °C
 B = 5.0% of salt concentration at 15 °C
 C = 2.5% of salt concentration at 30 °C
 D = 5.0% of salt concentration at 15 °C

Table 3 Effect of adding some nutrient components in MRS-B medium on bacteriocin Inhibitory activity (AU mm² mL⁻¹) by LAB isolates which isolated from yellow betung bamboo shoot pickle (C and D)

LAB Isolate	Pathogenic Bacteria	Activity Unit of Bacteriocins (mm ² mL ⁻¹)				
		Medium Compositions of Bacteriocin Production				
		MRS-B	MRS-B supplemented with glucose	MRS-B supplemented with glucose and tryptone	MRS-B supplemented with glucose and yeast extract	MRS-B supplemented with glucose, tryptone, and yeast extract
C18	1	-	323	831	537	226
	2	-	867	1061	353	-
	3	-	394	750	-	337
C19	1	-	-	173	-	-
	2	-	-	337	-	-
	3	-	-	415	-	-
C29	1	-	-	-	173	-
	2	-	-	-	293	-
	3	-	-	-	517	-
D11	1	-	1815	-	-	-
	2	-	2704	-	142	-
	3	-	2575	-	-	-
D20	1	-	-	-	-	-
	2	-	173	208	-	-
	3	-	94	77	-	0
D44	1	-	-	113	3179	795
	2	-	100	265	4663	3250
	3	-	-	245	3109	1558

Key: 1 = *L.monocytogenes* (FNCC 0156); 2 = *S.aureus* (FNCC 0047); 3 = *E.coli* (FNCC 0091)
 - = isolates showed no bacteriocin inhibitory activity against pathogenic bacteria

or yeast extract or a combination both of them). It had a broader zone of inhibition against pathogenic bacteria than other bacteriocins used in this study (Fig 1). The presence of 2% of glucose and 2% of yeast extract resulted in the largest bacteriocin inhibitory activity levels of 3179 AU mL⁻¹ against *Listeria monocytogenes* FNCC 0156, 4663 AU mL⁻¹ against *Staphylococcus aureus* FNCC 0047, and 3109 AU mL⁻¹ against *Escherichia coli* FNCC 0091 (Table 3 dan Fig 2).

LAB isolates had different response for each treatment of supplementations as shown on table 3. Carbon and nitrogen supplementation gave different response to induce bacteriocin inhibitory activity against *Listeria monocytogenes* FNCC 0156, *Staphylococcus aureus* FNCC 0047, and *Escherichia coli* FNCC 0091. Bacteriocin from isolate D44 showed the largest inhibitory activity against pathogens when it was grown in MRSB supplemented with 2% glucose and 2% yeast extract. Each isolate had different result on bacteriocin inhibitory activity for each supplementation. AU of 3179 mm² mL⁻¹ against *Listeria monocytogenes* FNCC 0156, 4663 mm² mL⁻¹ against *Staphylococcus aureus* FNCC 0047, and 3109 mm² mL⁻¹ against *Escherichia coli* FNCC 0091. Compared to the other tested pathogenic bacteria, the impact of this bacteriocin was less against *Escherichia coli* FNCC 0091 and *Listeria monocytogenes* FNCC 0156.

DISCUSSION

The inhibitory effect of LAB isolates may be due to the production of several antimicrobial compounds (Sifour *et al.* 2012). Different temperatures of fermentation may affect the potential of LAB in producing bacteriocins. Higher temperature altered the fermentation to homofermentative (Harris, 1998 in Salminen *et al.* 2004). Since the fermentation type of *Lactobacillus pentosus* is homofermentative (Fleming 1991 in Salminen *et al.* 2004), higher temperature (fermentation condition D) could be a better condition for the growth of *Lactobacillus pentosus* than fermentation condition B. Indirectly, maximum growth of *Lactobacillus pentosus* may affect its potential to produce bacteriocins. *Leuconostoc mesenteroides* belonged to heterofermentative bacteria showed a better bacteriocin inhibitory activity when isolated from higher temperature of fermentation (Fleming 1991 in Salminen *et al.* 2004).

Tryptone and yeast extract contain various amino acid which can help to increase the biomass (Salminen *et al.* 2004). The inhibition activity of bacteriocin against pathogens depends on nutrient addition (Table 3). Specific nutrients are required to improve bacteriocin production (Todorov and Dicks 2005b). Bacteriocins were only produced when nutrients were

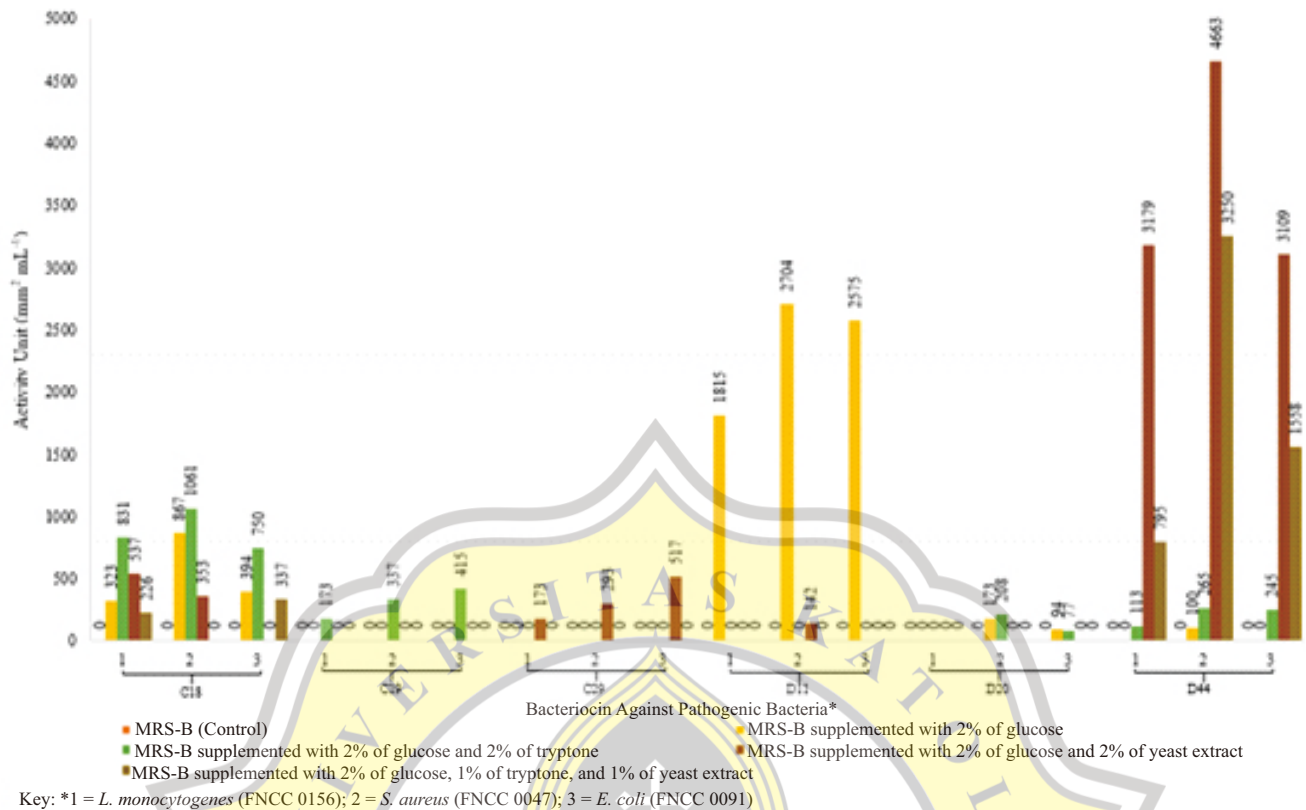


Fig 1 Effect of medium compositions on bacteriocin inhibitory activity (AU mm² mL⁻¹) of C and D isolates against *L. monocytogenes* (FNCC 0156), *S. aureus* (FNCC 0047), and *E. coli* (FNCC 0091).

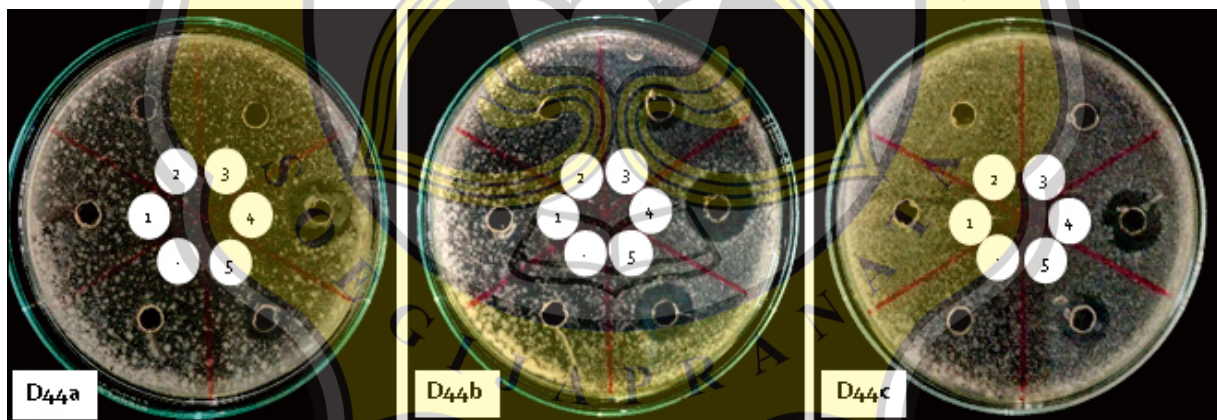


Fig 2 Effect of medium compositions on bacteriocin D44 inhibitory activity test using agar-well diffusion method against *L. monocytogenes* (FNCC 0156) (A), *S. aureus* (FNCC 0047) and *E. coli* (FNCC 0091) (C) ; Negative control (-) MRS-B (1) MRS-B supplemented with 2% of glucose (2) MRS-B supplemented with 2% of glucose and 2% of tryptone (3) MRS-B supplemented with 2% of glucose and 2% of yeast extract (4) MRS-B supplemented with 2% of glucose, 1 % of tryptone and 1% of yeast extract (5).

available during the incubation period. It has been proved that the composition of the growth medium is very important for the production of individual bacteriocins (Todorov and Dicks 2005b).

Yeast extract is a rich source of vitamin and provides excellent growth conditions for more microorganisms. It is probable that the yeast extract may take a part in deactivation of an inhibitor of

bacteriocin synthesis (Fukushima *et al.* 1983). It was observed that all bacteriocins had different inhibitory effect on *Listeria monocytogenes* FNCC 0156, *Staphylococcus aureus* FNCC 0047, and *Escherichia coli* FNCC 0091. Surprisingly, the same LAB species from different fermentation conditions had different bacteriocin inhibitory activity (Table 1).

Some bacteriocins inhibited not only pathogenic

but also Gram-positive and Gram-negative in general. Bacteriocins are ribosomally synthesized compounds produced by bacteria in order to inhibit the growth of other bacteria. Bacteriocins have a narrow killing spectrum and thus they are generally able to kill only bacteria closely related to the producing strain (Cleveland *et al.* 2001 in Salminen *et al.* 2004). Moreover, Gram-negative bacteria was mostly resistant to the bacteriocins of LAB. Bacteriocin produced by *Lactococcus lactis* KCA2386 and plantaricin 35d produced by *Lactobacillus plantarum* had inhibitory activity against Gram-negative bacteria (Ivanova *et al.* 2000). This is quite different from most of the bacteriocins which inhibit only closely related strains (Hata *et al.* 2010).

Inhibitory effects of bacteriocin on tested bacteria vary (Table 3). The differences between the inhibition zone against each pathogenic bacteria associated with the components of the cell structure. Inhibition mechanism by antibacterial compounds is by destroying the bacterial cell wall. The response of LAB in damaging the peptidoglycan component is stronger on neighbor cells (Gram-positive bacteria). Lipopolysaccharide (LPS) layer on Gram-negative bacteria cells can interfere the ability of bacteriocin to inhibit Gram-negative pathogenic bacteria. Some exceptions were found, bacteriocin of C19 and C29 had a broader inhibitory activity against *Escherichia coli* FNCC 0091 than *Staphylococcus aureus* FNCC 0047 and *Listeria monocytogenes* FNCC 0156. This exceptions could have occurred because of original growth condition of isolates and bacteriocin diffusion on agar-media.

In conclusion, carbon and nitrogen supplementation were able to induce the production of bacteriocin in some LAB. Bacteriocin of LAB from pickled Yellow Betung bamboo shoots shows inhibition towards *Listeria monocytogenes* FNCC 0156, *Staphylococcus aureus* FNCC 0047, and *Escherichia coli* FNCC 0091.

ACKNOWLEDGMENT

This work was supported by a grant from Directorate General of Indonesian Higher Education. This research a part of the second year of Penelitian Unggulan Perguruan Tinggi (PUPT) 2016/2017 entitled "Efek Probiotik dan Mikrostatik dari Bakteri Asam Laktat yang Berperan dalam Fermentasi Acar Rebung", grant number 011/K6/KM/SP2H/RISET TERAPAN/2016.

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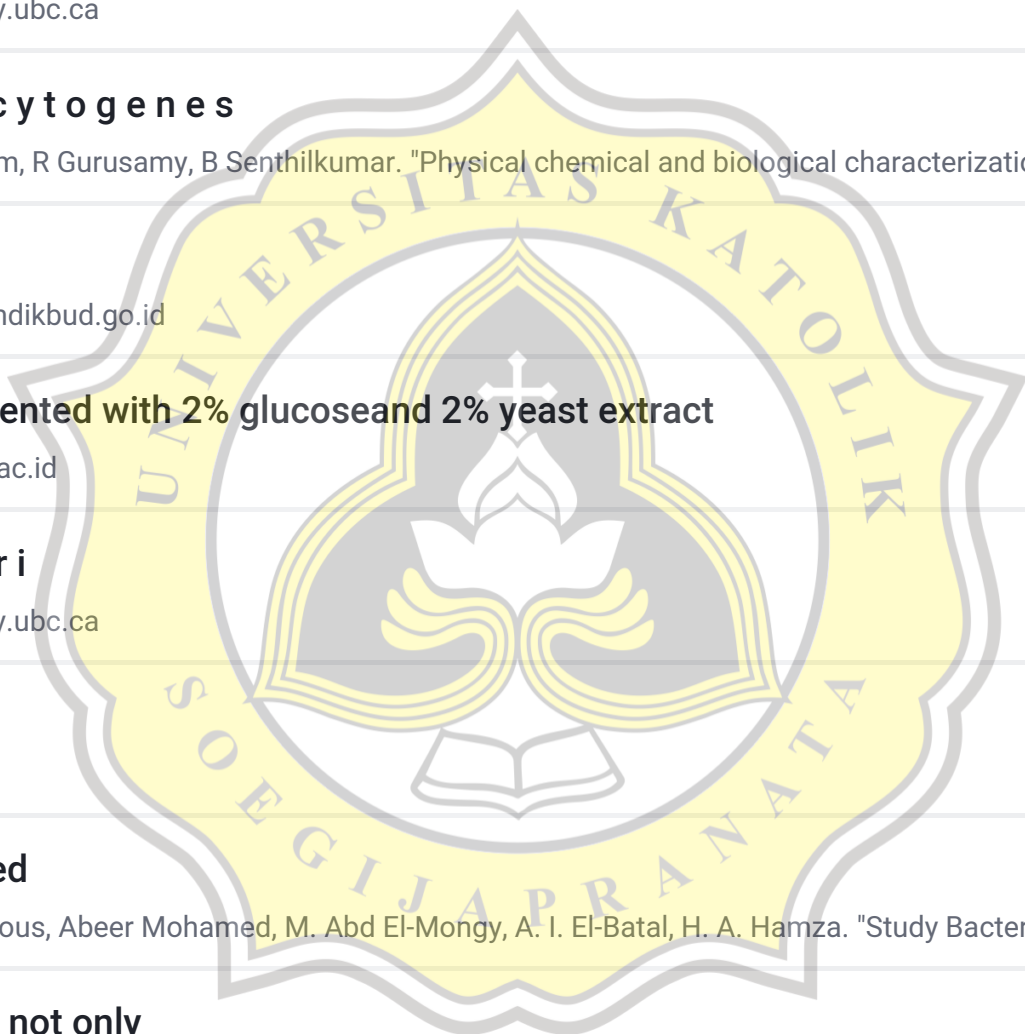
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