

Characterisation of crude bacteriocin produced by *Pediococcus pentosaceus* 2A2 in enriched molasses medium

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Abstract

The objectives of the present work were to determine the optimal molasses concentration as carbon source in enriched molasses medium for bacteriocin production by *Pediococcus pentosaceus* 2A2, and to evaluate the characteristic of bacteriocin produced. Enriched molasses medium was based on MRS (de Man Rogosa and Sharpe) broth, and molasses was used instead of glucose as carbon source. Bacteriocin production was performed in enriched molasses media containing various molasses concentrations (10% to 50%) added with 0.5% meat extract, 3% yeast extract, 1% peptone, Tween 80 and minerals, and incubated at 37°C for 24 h. The cell-free supernatants (CFS) of the culture were further examined for antibacterial activities against *Listeria monocytogenes* ATCC 7644, expressed as the diameter of inhibition zone using the disc diffusion method. The results showed that CFS produced in medium containing 30% molasses demonstrated high antibacterial activity but was not significantly different as compared to the control medium. The CFS was then precipitated using gradient concentration (20% to 90%) of ammonium sulphate, centrifuged (12,000 g, 15 min, 4°C), followed by dialysis through 2.0 kDa cut off membrane to obtain crude bacteriocin for characterisation study. Crude bacteriocins produced in enriched medium containing 30% molasses and MRS broth were stable either to heat treatments (30 min at 80°C, 15 min at 100°C and 121°C) or pH (2.0; 4.0 and 6.0). The present work gave evident that molasses have the potential to be used as low-cost component for bacteriocin production by *P. pentosaceus* 2A2. In addition, as compared to sodium benzoate and sodium nitrite, the antibacterial activity of crude bacteriocin against tested bacteria was also significantly higher, indicated that it may be promising to be applied in food.

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Keywords

Pediococcus pentosaceus
2A2
Bacteriocin
Molasses
Characterisation

Introduction

The awareness on food safety among the population has increased recently. The consumers prefer natural instead of synthetic preservatives. Therefore, it is necessary to explore other alternative such as natural preservative, including metabolites produced by microorganisms (i.e., bacteriocin; Savadogo *et al.*, 2006). Nisin for example, which is produced by *Lactococcus lactis* subsp. *lactis*, is the first commercialised bacteriocin, marketed as Nisaplin™, and has been authorised as a food preservative since 1969 (Deegan *et al.*, 2006). Nisin is effective against Gram-positive bacteria (Rodriguez *et al.*, 2003), and the use of nisin has been approved by many countries including Indonesia (Standards Indonesia, 2013). Other bacteriocin that

has also been successfully commercialised as a food bio-preservative is pediocin PA-1 produced by *P. acidilactici*, and marketed as ALTA™ 2431 (Deegan *et al.*, 2006). It has been reported as anti-listeria with a broad pH range and stable to heat treatment (Rodriguez *et al.*, 2003). Arief *et al.* (2015) found one candidate strain of *Pediococcus pentosaceus* 2A2 isolated from Indonesian beef that produced bacteriocin. The identification of *Pediococcus pentosaceus* 2A2 had been successfully done by 16S rRNA gene sequencing. However, the characterisation of this bacteriocin has not been done yet.

Studies on bacteriocin production were usually conducted using MRS (de Man Rogosa and Sharpe) broth (Alvarez *et al.*, 2004; Todorov, 2008; Castro *et al.*, 2011) which is considered relatively expensive for commercialisation purpose. One example of low-

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cost waste product is molasses which can be used for the cultivation of LAB and had been reported as able to support the growth of *Lactobacillus plantarum* AMA-K, and demonstrated comparable growth rate and bacteriocin production as compared to MRS broth (Todorov, 2008).

The objectives of the present work were to determine the optimal molasses concentration as carbon source in enriched molasses medium for bacteriocin production by *Pediococcus pentosaceus* 2A2, and to evaluate the characteristic of bacteriocin produced.

Materials and methods

Bacterial strains and media

Pediococcus pentosaceus 2A2 was maintained in MRS broth (Oxoid, UK) at 37°C. Indicator bacteria (*Listeria monocytogenes* ATCC 7644 and *Staphylococcus aureus* ATCC 25923) were used to test the antibacterial activity, and were grown in Trypticase Soy Agar (Oxoid, UK) at 37°C. Molasses were obtained from the sugar cane industry (PT Rajawali Subang, West Java, Indonesia) which consisted of 6.68% glucose, 0.4% nitrogen, 1.14% potassium, 664.18 mg/kg calcium, 1,241.89 mg/kg magnesium, 274.55 mg/kg phosphorus with 65.72% moisture content. No heavy metal was detected.

Determination of incubation time for bacteriocin production by P. pentosaceus 2A2

MRS broth (100 mL) in different tubes were inoculated with 10% (v/v) 24 h culture of *P. pentosaceus* 2A2 (108 CFU/mL), and incubated at 37°C for 36 h. At every 4 h interval, the sample was measured for pH and antibacterial activity. Antibacterial activity of neutralised cell-free supernatant was analysed by the disc diffusion method against *L. monocytogenes* ATCC 7644 and *S. aureus* ATCC 25923, and expressed as diameter of inhibition zone. The optimum incubation time for bacteriocin production was determined by the largest inhibition zone observed.

Bacteriocin production in various concentrations of enriched molasses media

Enriched molasses media were prepared based on the composition of MRS broth, except for glucose which was replaced with molasses at various concentrations of 10% to 50%. All experimental media were enriched with 3% yeast extract, 1% peptone, 0.5% meat extract, 0.2% dipotassium hydrogen phosphate, 0.1% Tween 80, 0.2% ammonium citrate, 0.2% sodium acetate, 0.02% magnesium sulphate

and 0.005% manganese sulphate. MRS broth as control medium was also enriched with 3% yeast extract (Arief *et al.*, 2013). All media were adjusted to pH 6.2 using 1 N NaOH, and then inoculated with 10% (v/v) 24 h culture of *P. pentosaceus* 2A2 and incubated at 37°C for 24 h. Cells were harvested by centrifugation at 12,000 g for 15 min at 4°C, and the supernatant was evaporated at 60°C for 1 h before adjusted to pH 6.8 using 1 N NaOH to exclude the antimicrobial effect of organic acids (Todorov and Dick, 2009). The supernatant was passed through a membrane filter (0.22 µm pore size) to obtain the CFS, and analysed further for its antibacterial activity against *L. monocytogenes* ATCC 7644 and *S. aureus* ATCC 25923 using the disc diffusion method. The medium containing molasses with the largest inhibition zone was further assessed.

Production and characterization of crude bacteriocin

P. pentosaceus 2A2 were grown in the selected media as previously described and centrifuged at 12,000 g for 15 min at 4°C. The supernatant was further evaporated at 60°C for 1 h, adjusted to pH 6.8 and passed through 0.2 µm membrane filter (Millipore) to obtain the CFS. The CFS was then added with gradient concentrations of ammonium sulphate (20%, 40%, 60%, 80% and 90%), stirred gently for 2 h at 4°C and then centrifuged (12,000 g, 15 min, 4°C). The precipitate was resuspended in sterile potassium phosphate buffer and dialysed through 2.0 kDa cut-off membrane to obtain crude bacteriocin. The antibacterial activity of crude bacteriocin was further examined against *L. monocytogenes* ATCC 7644 and *S. aureus* ATCC 25923.

Antibacterial activity assay

The assay for antibacterial activity of bacteriocin was conducted by the disc diffusion method according to Dhiman *et al.* (2011) with slight modification. Sterile paper discs (5 mm diameter, Oxoid) containing approximately 50 µL crude bacteriocin was placed on Muller Hinton Agar (Oxoid, UK) plate on which surface had been spread (separately) 0.1 mL 24 h culture of *L. monocytogenes* ATCC 7644 and *S. aureus* ATCC 25923 (108 CFU/mL), then incubated at 37°C for 24 h. The antibacterial activity of bacteriocin was expressed as diameter of clear zone (mm) formed around the paper discs.

Stability of crude bacteriocin at different temperature and pH

To investigate the effect of temperature, the crude bacteriocin was heated at 80°C for 30 min, and

100°C and 121°C for 15 min (Arief *et al.*, 2013). To investigate the effect of pH on antibacterial activity, the pH of the crude bacteriocin was adjusted to 2.0, 4.0 and 6.0 with either 1 N HCl or 1 N NaOH, and incubated for 1 h at room temperature. Following incubation, the samples were readjusted to pH 6.8 using 1 N NaOH (Altuntas *et al.*, 2010). After exposing at different temperature and pH, the stability of crude bacteriocin were tested using the disc diffusion method against *L. monocytogenes* ATCC 7644 and *S. aureus* ATCC 25923.

Mode of action of crude bacteriocin activity

Ten milliLitre of TSB was inoculated with 1% (v/v) 24 h culture of *L. monocytogenes* ATCC 7644 and *S. aureus* ATCC 25923 (separately), and incubated for 3 h at 37°C. Next, 1 mL crude bacteriocin was added to the culture and changes in OD₆₀₀ were recorded hourly over a 10-h period (Todorov *et al.*, 2012). The mode of action of bacteriocin could be considered as bacteriostatic if the bacteriocin inhibited the growth of the tested bacteria, and bactericidal if it killed the tested bacteria during incubation, which was confirmed further by plating method (Todorov *et al.*, 2012).

Potential activity of crude bacteriocin to synthetic preservatives

This test was performed according to Arief *et al.* (2012). The inhibitory activity of crude bacteriocin was compared to two concentrations of sodium nitrite (30 and 300 ppm) and sodium benzoate (1,000 ppm) against *L. monocytogenes* ATCC 7644 and *S. aureus* ATCC 25923 by the disc diffusion method as described above.

Statistical analysis

All experiments were conducted in duplicates and mean values were analysed by Analysis of Variance followed by Least Significant Difference at 95% confidence level (Steel and Torrie, 1995).

Results and discussion

Effect of incubation times on antibacterial activity

The antibacterial activities of CFS of *P. pediococcus* 2A2 were observed in MRS broth in order to determine the effective length of time for bacteriocin production, expressed as diameter of inhibition zones (including diameter of paper disc), and are presented in Table 1. The largest inhibition zone was achieved by CFS at 24 h incubation (11.53 ± 0.47 mm) and then decreased at 28 h up to 36 h. Based on this result, 24 h incubation was selected

as the effective length of time for crude bacteriocin production in further experiments. Bacteriocin production mostly occurs in early exponential growth phase and reached maximum inhibitory activity at the beginning of the stationary phase (Bagenda *et al.*, 2008; Huang *et al.*, 2009; Kingcha *et al.*, 2012; Abrams *et al.*, 2013; Mandal *et al.*, 2014).

Table 1. Antibacterial activity of the cell-free supernatant of *P. pentosaceus* 2A2 against *L. monocytogenes* ATCC 7644 during incubation in MRS broth enriched with 3% yeast extract at 37°C

Time of incubation (h)	Zone of inhibition (mm)*
1	7.14 ± 0.40a
4	8.11 ± 0.40b
8	8.21 ± 0.02 b
12	8.54 ± 0.40 b
16	8.55 ± 0.03 b
20	9.86 ± 0.34 c
24	11.53 ± 0.47 d
28	10.12 ± 0.09 c
32	7.67 ± 0.16 ab
36	6.87 ± 0.33 a

*Values are expressed as means + standard deviation

Average value with different superscripts in the same column indicate significant differences ($p < 0.05$)

Effect of various molasses concentrations on antibacterial activity

Generally, CFS produced in all media containing molasses with different concentrations (10% to 50%) exhibited inhibition zones against *L. monocytogenes* ATCC 7644 with diameter range of 7.73 mm to 9.88 mm (Figure 1). Largest inhibition zones (9.74 mm to 9.88 mm) by CFS were produced in 30% and 40% molasses-enriched medium which were not significantly different as compared to control. For efficiency reason, medium containing 30% molasses was selected for further crude bacteriocin production.

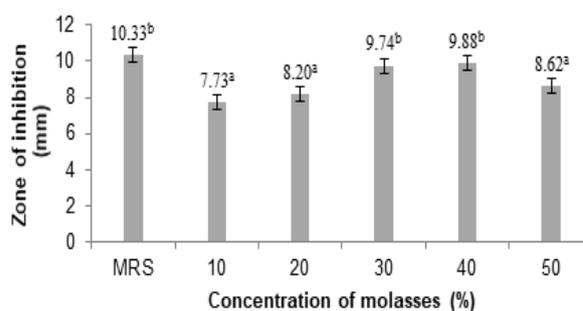


Figure 1. Antibacterial activity of cell-free supernatant produced by *P. pentosaceus* 2A2 in enriched media containing various concentrations of molasses against *L. monocytogenes* ATCC 7644

Average values with different superscripts indicate significant differences ($p < 0.05$)

Similar to CFS, the partially purified crude bacteriocin also showed antibacterial activity against *L. monocytogenes* ATCC 7644 and *S. aureus* ATCC 25923 which were not significantly different as compared to control sample. The crude bacteriocin exhibited relatively higher inhibition zone against *L. monocytogenes* ATCC 7644 (10.86 ± 0.21 mm and 10.10 ± 0.33 mm) when compared to *S. aureus* ATCC 25923 (9.87 ± 0.32 mm and 9.77 ± 0.06 mm; data not shown), for bacteriocin produced in enriched-medium containing 30% molasses and control medium, respectively. Other strain of *P. pentosaceus* ST44AM produced relatively high inhibition zone (28 mm) against *L. monocytogenes* Scott A (Todorov and Dick 2009). This difference might be due to the different strains of bacteriocin producers, indicator pathogen used, different inhibition assay and bacteriocin purification method. Pal *et al.* (2010) also reported that bacteriocin produced by *P. pentosaceus* JJ1 exhibited higher activity against *L. monocytogenes* and *S. aureus* as compared to bacteriocin produced by *P. pentosaceus* JJ6.

Concentration of bacteriocin crude extract

The protein concentration of bacteriocin produced in enriched-molasses medium was 11.38 ± 0.14 mg/mL which was higher than that in control medium (8.12 ± 0.14 mg/mL). Pal *et al.* (2010) reported that the protein concentration of bacteriocin JJ1 produced by *P. pentosaceus* JJ1 was 4.65 ± 0.61 mg/mL, and bacteriocin JJ6 was 4.12 ± 0.44 mg/mL. Beside the different strain used, the difference in protein concentrations might be due to the purity of bacteriocin. In the present work, the bacteriocin was only partially purified through ammonium sulphate precipitation followed by dialysis, while Pal *et al.* (2010) performed further purification through Gel Permeation Chromatography (GPC).

Stability of bacteriocin to heat treatment and acidity

The stability of bacteriocin to high temperature and low pH were observed based on the inhibition activity against *S. aureus* and *L. monocytogenes* (Table 2). The antimicrobial activity of bacteriocins either produced in enriched-molasses medium or control medium were found to be heat stable after heating at 80°C for 30 min and 100°C-121°C for 15 min against both tested bacteria with the range of inhibition zones of 7.50 - 9.67 mm. The bacteriocins remained active following incubation for 1 h at pH 2.0, 4.0 and 6.0 with the range of inhibition zone of 7.33 - 8.77 mm. These results demonstrated that the stability towards high heat and low pH of the bacteriocins produced in enriched-molasses medium was comparable to that produced in control medium. The stability of bacteriocin to high heat and low pH were also reported by other researchers (Huang *et al.*, 2009; Pal *et al.*, 2010; Todorov *et al.*, 2011 and Mandal *et al.*, 2014), which justify the application of bacteriocin during the thermal-processing of foods. According to Drider *et al.* (2006), small bacteriocins (< 10 kDa) are generally heat-resistant.

Mode of action of bacteriocin activity

The mode of action of bacteriocin was observed by measuring the growth of *L. monocytogenes* ATCC 7644 and *S. aureus* ATCC 25923 over 10-h period. Changes of the OD₆₀₀ of both indicator bacteria are shown in Figure 3. The growth of *L. monocytogenes* ATCC 7644 and *S. aureus* ATCC 25923 without the addition of crude bacteriocin increased from OD₆₀₀ 0.2 to 1.8 and from OD₆₀₀ 0.2 to 2.0, respectively. After the addition of crude bacteriocin, the growth of *L. monocytogenes* ATCC 7644 and *S. aureus* ATCC 25923 were inhibited as shown by lower OD₆₀₀ either for *L. monocytogenes* ATCC 7644 or for *S. aureus* ATCC 25923. Higher inhibition activities (lower

Table 2. Effect of different temperatures and pH's on the stability of bacteriocin activity (mm zone of inhibition) produced in enriched medium containing 30% molasses and control medium towards indicator bacteria

Treatment	Zone of inhibition (mm) towards <i>S. aureus</i>		Zone of inhibition (mm) towards <i>L. monocytogenes</i>	
	A	B	A	B
Control	9.77 ± 0.06^a	9.87 ± 0.32^a	10.10 ± 0.33^a	10.86 ± 0.21^a
80°C 30 min	9.47 ± 0.26^b	9.67 ± 0.25^{ab}	8.22 ± 0.22^b	8.08 ± 0.30^b
100°C 15 min	9.05 ± 0.86^b	8.76 ± 0.11^b	9.22 ± 0.56^c	8.28 ± 0.44^b
121°C 15 min	7.50 ± 0.15^c	9.43 ± 0.48^c	8.18 ± 0.41^b	7.91 ± 0.08^b
pH 2.0	7.33 ± 0.12^b	7.92 ± 0.14^b	7.60 ± 0.13^b	7.72 ± 0.07^b
pH 4.0	7.72 ± 0.16^a	8.65 ± 0.14^a	8.24 ± 0.32^c	7.91 ± 0.03^c
pH 6.0	7.82 ± 0.18^a	8.77 ± 0.15^a	8.64 ± 0.15^d	8.04 ± 0.06^c

A : bacteriocin produced in MRS supplemented with 3% yeast extract

B: bacteriocin produced in enriched medium containing 30% molasses

Average values with different superscripts in the same column indicate significant differences ($p < 0.05$)

OD₆₀₀) were demonstrated by the bacteriocin obtained from enriched-molasses medium on the growth of both tested bacteria (Figure 2). Viable counts of bacteriocin-treated *L. monocytogenes* ATCC 7644 and *S. aureus* ATCC 25923 examined at 10 h were relatively low, only at 100 CFU/mL. These results suggest that bacteriocin produced by *P. pentosaceus* 2A2 has a bacteriostatic mode of action, instead of bacteriocidal. Similar findings were reported for bacteriocin ST211CH (Todorov *et al.*, 2012), bacteriocin ST44AM (Todorov and Dicks, 2009) and bacteriocin PPK34 (Abrams *et al.*, 2013).

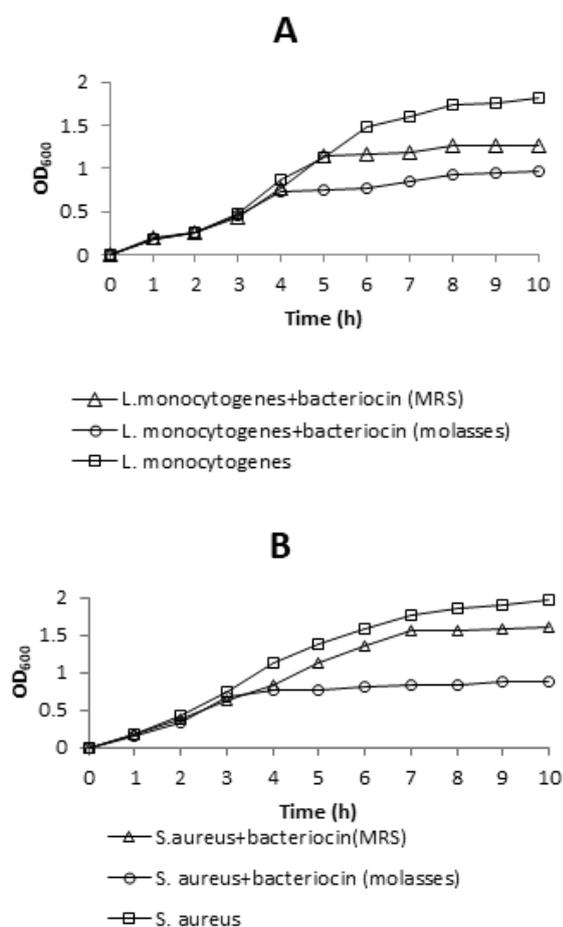


Figure 2. Growth of *L. monocytogenes* ATCC 7644 (A) and *S. aureus* ATCC 25923 (B) in the presence or absence of crude bacteriocin produced in 30% molasses-enriched medium and control medium (MRS broth + 3% yeast extract) Potential activity of crude bacteriocin compared to chemical preservatives

The inhibition zone formed by crude bacteriocin produced either in enriched-medium containing 30% molasses or control medium were relatively greater (9.77 ± 0.06 mm to 10.86 ± 0.21 mm) as compared to that with chemical preservatives such as sodium benzoate 1,000 ppm and 30 and 300 ppm sodium nitrite (5.09 ± 0.10 mm to 8.10 ± 0.13 mm). These

results showed that the bacteriocin produced by *P. pentosaceus* 2A2 has potential application in food as replacement for chemical preservatives.

Conclusions

The optimal incubation time for bacteriocin production by *P. pediococcus* 2A2 with largest inhibition zone against *L. monocytogenes* was observed after 24 h, and 30% molasses added into the medium as carbon source was found to be optimum for bacteriocin production by *P. pentosaceus* 2A2 with comparable antibacterial activities to control medium against *L. monocytogenes* ATCC 7644 and *S. aureus* ATCC 25923. Other characteristics of crude bacteriocin obtained from enriched-medium were also comparable to control medium including heat and acid resistance. Compared to chemical preservatives, the antibacterial activity of crude bacteriocin against *L. monocytogenes* and *S. aureus* was relatively higher than sodium benzoate and sodium nitrite which showed potential use of the bacteriocin as a replacement for chemical preservatives in foods. However, further work needs to be conducted by using pure bacteriocin.

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