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Production and Extraction Of Antibacterial Bacteriocin from *Pediococcus sp.* NWD 015

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Abstract

The aim of this Research was to study the capability of *Pediococcus sp.* NWD 015 to produce bacteriocin. Specific objectives were to study the growth pattern of *Pediococcus sp.* NWD 015 and bacteriocin activity, extraction and characterization of bacteriocin, and to determine the effect of storage time and temperature on bacteriocin activity. Results showed that the bacteriocin activity increased during growth and reached the highest activity during stationary phase. The maximum bacteriocin production reached after incubation of the cell for 12 h at 37°C in TGE broth and decreased after 96 h incubation. Extraction with adsorbtion-desorbtion method could increased a specific activity of bacteriocin. Bacteriocin from *Pediococcus sp.* NWD 015 is inactivated by Proteinase-K; however it is still active by heat treatment at 121°C for 15 min and over pH 2 – 11. Bacteriocin of *Pediococcus sp.* NWD 015 was effective againts *Enterococcus faecalis*, *Staphylococcus aureus*, *Eschericia coli*, *Listeria monocytogenes* but not against *Salmonella thypimurium*. The molecular weight of bacteriocin is 4.95 kDa.

Keywords : *Bacteriocins, Pediococcus sp NWD 015.*

Introduction

The trend of food processors is to produce foods that are minimally processed (believed by some to be more "natural") may in fact compromise or eliminate previous product safeguards. Minimally, the food processing has to be attractive to the consumer because natural, fresh appearance, viewed as nutritionally correct, and do not contain preservatives. Elimination of preservatives from perishable foods and the subsequent necessary dependence on refrigeration as the primary preservation mechanism may be a risk. It has generally been assumed that refrigerated foods are adequate to prevent the growth of pathogenic and toxigenic microorganisms which are associated with foods. This assumption is no longer valid, pathogens such as *Listeria monocytogenes* can growth at temperature abuse during commercial handling compounds the problem. Thus, food processing are faced with the dilemma of manufacture attractive, minimally chemical preservatives on foods, and to ensure a safe product (Daeschel, 1993; Leveau *et al.*, 1995; Jack *et al.*, 1995).

Lactic acid bacteria produce metabolites that are inhibit toward other microorganisms. Historically, lactic fermentations as a preservation process which is based in part upon such metabolites accumulating to inhibitory levels in certains food and beverages. The antimicrobial metabolites produced during fermentations include lactic and acetic acids, ethanol, hydrogen peroxide, and bacteriocins. Bacteriocins are biologically active proteins or protein complexes displaying a bactericidal mode of action exclusively

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towards gram-positive bacteria and particulary closely related species (Daeschel, 1992). In addition to the use of bacteriocins to control food-borne pathogens, they have potential application in controlling bacterial spoilage of food and in directing pure culture fermentations in foods and beverages. This research was to study the capability of *Pediococcus sp*. NWD 015 to produce bacteriocin and specificity are to study the growth pattern of *Pediococcus sp.* NWD 015 and bacteriocin activity, extraction and characterization of bacteriocin, and determination of molecule mass.

Materials and Methods

Bacterial cultures

The strain *Pediococcus sp.* NWD 015, provided by Umami *et al.*, 2004 was maintained at -40°C as frozen stock cultures in equal volumes of 10% skim milk and 10% glycerol. *Pediococcus sp.* NWD 015 and *Enterococcus faecalis* were grown in trypticase glucose yeast extract (TGE) broth, *Staphylococcus aureus* FNCC 0047, *Listeria monocytogenes* ATCC 7644, *Eschericia coli* FNCC 0091, and *Salmonella thypimurium* FNCC 0050 were grown in nutrient broth (NB). The cultures were grown at 37°C for 24 h in TGE or NB medium. As necessary, 1.5 % agar was added to TGE and NB to make a solid medium.

Rejuvenation of culture of bacteria was carried out to the fresh media. The purity test was done by microscopie and gram staining observation. Identification and characterization of *Pediococcus sp.* NWD 015 were performed by methods which are described by Yang *et al.* (1992) and Ray *et al.*, (1997), including observation on the characteristics of size and gram stainning, the biochemical, the physiology test, the type of fermentation, and the type of the lactate.

The analysis of the growth patterns from *Pediococcus sp.* NWD 015 was done by measuring the growth and the bacteriocin production for 24 h using spectrophotometer (600 nm) and disc assays.

Preparation of crude bacteriocin and antimicrobial activity assay

Pediococcus sp. NWD 015 was grown in 1% (v/v) TGE broth for 24, 48, and 96 h at 37°C and was divided into 50 ml fractions. The supernatants were harvested by centrifugation at 29,000xg for 15 min. These samples were adjusted to pH 6.5 with sterile NaOH. The samples were heated to 100°C for 25 - 30 min and supernatant was neutralized, filtered through at 0.2 mm membrane filter (Biswas *et al.*, 1991*)*.

An aliquot of a test culture broth was heated in boiling water for 30 min and serially diluted, and 100 ìl from each dilution was spotted onto a well in an assay plate. The assay plate had a layer of TGE hard agar seeded with indicator strains (10^8). The plates were incubated at 37°C for overnight, and the highest dilution that produced a distinct zone was multiplied by 10 to obtain the activity units per milliliter (1 AU was defined as the amount of bacteriocin producing 1 mm^2 of inhibition area around the steril disc).

Characterization of bacteriocin

(a). Effect of enzymes on antimicrobial activity.

To determine the sensitivity of *Pediococcus sp.* NWD 015 to enzyme proteolytic. The enzymes (*Proteinase*-K) were dissolved in sterile 4 mM/l phosphate buffer, pH 7.0, at a concentration of 200 ug/ml. Supernatant was added to the enzyme solutions at a concentration of 10 mg/ml and the samples were incubated at 37°C for 1 h. The activity of the sample was then determined by disc assay method.

(b). Effect of heat treatment on antimicrobial activity.

For thermal stability determination of *Pediococcus sp.* NWD 015, supernatant free cell (bacteriocin) was boiled at 100°C for 10, 20, and 30 min. Another sample was

autoclaved (121°C) for 15 min, and all samples were cooled and assayed for its activity.

(c). Effect of pH on antimicrobial activity.

Bacteriocins from *Pediococcus sp.* NWD 015 was dissolved in deionized water. Samples from this were adjusted with sterile 10 mM / l NaOH or 10 mM / l HCI to different pH levels between 2 to 11. Samples were maintained for 30 min at 100°C. The samples were then adjusted to pH 6.5 with dH_2O sterile and assayed for its activity.

(d).Determination the effect of storage time and temperature on bacteriocin activity.

Bacteriocin from *Pediococcus sp.* NWD 015 was storage at 37°C (control), 4°C and -20°C for 10 days and the activity of the sample was then determined by disc assay method.

Extraction of bacteriocin (adsorbtion-desorbtion method)

Pediococcus sp. NWD 015 was grown to early stationary phase in TGE broth. Broth culture was heated at 100°C for 30 min to kill the cells then adjusted pH to 6.5. The cells were harvested by centrifugation at 15,000xg for 15 min. After the cells had been washed with 2 mM Na_2HPO_4, then resuspended in 0.1 M NaCI and the pH of the mixture was adjusted to 2.0; 2.5; and 3.0. The mixture was kept at 4°C for 24 h by mixing with stirrer. Cell suspensions were then centrifuged at 29,000xg for 30 min, and the supernatants were dialyzed in 3500 MW cut-off spectra with dH_2O at 4°C 24 h (Yang *et al.*, 1992; Naclerio *et al.*, 1993; Wardani, 1999).

Determination of bacteriocin molecular weight

The supernatants of *Pediococcus sp.* NWD 015 were fractionated by 12% SDS PAGE after treated in sample buffer (10% SDS, gliserol, 2-mercaptoethanol, brilliant blue G, Tris-HCl, pH 6.8). The electrophoresis was performed at 80 volt for approximately 30 min and then 100 volts for another 1.5 h. After that, the gel was removed and cut into two vertical parts. One part of the gel, containing samples and moleculer weight standards (Biorad), was stained with coomasie brilliant blue G and the other part was tested for antimicrobial activity by using growth inhibition of *S. aureus* with the following modifications. The gel was then placed into a petri dish and overlaid with 5 ml TGE of 1.5 % agar containning 10^7 cells of the indicator strain. The dish was then incubated at 37°C for about 24 h and analyzed for an inhibition halo (Bhunia *et al.*, 1992; Naclerio *et al.*, 1993; Osmanagaoglu *et al.*, 1998).

Results and Discussion

Growth and production of bacteriocin by Pediococcus sp. NWD 015 in a liquid TGE medium at 37°C

The growth and bacteriocin production of *Pediococcus* sp. NWD 015 is presented in Table 1 and Figure 1 and 2. A slight increase of cell dry weight was observed for 24 h of fermentation. The cells were rapidly increased after 5 h of fermentation and then relatively constant until the end of fermentation. This phase was considered as log phase and followed by stationary phase.

Table 1. The bacteriocins activity (AU / ml) from *Pediciococcus* sp. NWD 015

Hours	Bacteriocin activity (AU/ml)
3	Not identified
4	100
6	100
8	300
10	400
11	500
12	600
13	600
14	600
15	600
18	600
48	600
96	500

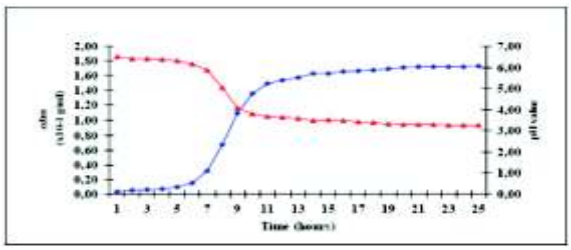

Figure 1. The growth patterns of *Pediococcus sp.* NWD 015 and the pH change over 24 hours

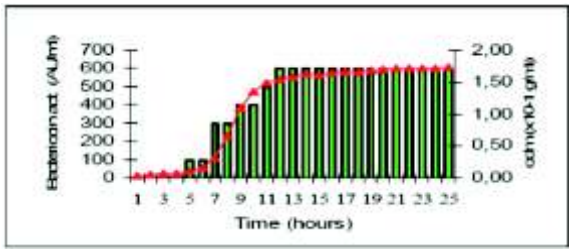

Figure 2. Production of bacteriocin by *Pediococcus sp.* NWD 015 for 24 h of fermentation (AU / ml)

The pH of the fermentation medium slightly decreased during the first four hour of fermentation, then sharply decreased during log phase. The pH was then relatively constant for the rest of the fermentation period. During log phase (6 – 11 h fermentation), level pH of the medium decreased rapidly which was coincident with the increase in the cell dry weigt. The data indicated that pH of the medium was inversely proportional with growth of *Pediococcus sp.* NWD 015.

The bacteriocin was initially produced since the first 4 h of fermentation and has an optimal activity for 12 h of fermentation. Bacteriocin activity remain stable up to 48 h of fermentation, then its drop in the activity after 96 h incubation of fermentation table 1. The bacteriocin activity was declined after incubation for 96 h which its was predictedly because of the following reasons: (i) bacteriocin synthesis stopped along with the end of synthesis of DNA and RNA, (ii) enzymes involved in processing of prebacteriocin were not present all the time but were synthesized for a brief period towards the end of growth phase, and (iii) the existence of protease / proteolitic enzymes reduce the bacteriocin activity (Biswas *et al.*, 1991; Hoover and Harlander, 1993). Therefor this results suggest that 12 h incubation is a good condition for bacteriocin production.

Antimicrobial activity assay (Sensitivity to pathogen bacteria)

The inhibitory spectrum of bacteriocins against several Gram-positive and Gram-negative microorganisms such as *Listeria monocytogenes*, *Eschericia coli*, and *Salmonella thypimurium* were done in this research (Table 2, Figure 3). This is an important characteristic in order to evaluate the possibility of using the bacteriocin-producing strains as an additional barrier against spoilage and / or food-borne microorganisms in foods. Bacteriocin produced by *Pediococcus sp.* NWD 015 showed effective againts *Listeria monocytogenes* (300 AU / ml) and *E. coli* (400 AU / ml) but not againts *Salmonella thypimurium*.

Table 2. The Sensivity bacteriocin of Pediococcus sp. NWD 015 to pathogen bacterias such as Listerya monocytogenes. Salmonella thypimurium, and E. coli.

Indicator	Bacteriocin activity (AU/ml)
Listeria monocytogenes	300
Eschericia coli	400
Salmonella thypimurium	-

Sensitivity of *E. coli* as gram-negative bacterial cells to bacteriocin is a consequence of sublethal injury in the outer membrane of the cells that allows bacteriocin to pass through the outer membrane and come in contact with the cytoplasmic membrane and destabilize its functions, but not in *Salmonella thypimurium*. It was expected that *Salmonella thypimurium* had peptide compositions of peptidoglycan on cell membrane (Berge, 1992; Jack *et al.*, 1995; Leveau *et al.*, 1995) as different as *E. coli*, so as the capacity of molecules bacteriocin that was hydrofobic

not or seldom could be gather to the cluster of carboxyl from acid diamino to peptidoglycan.

Note : A. *Eschericia coli* (400 AU/ml), B. *Listeria monocytogenes* (300 AU/ml), C. *Salmonella thypimurium* (no activity)

Figure 3. Bacteriocin activity againts pathogen bacterias

Characterization of bacteriocin produced by Pediococcus sp. NWD 015

(a). Sensitivity to protease.

Bacteriocins are by definition proteinaceous substances, must be sensitive to at least one proteolytic enzyme. Consequently, protease sensitivity is a key criterion in its characterization. In this study, the bacteriocin produced by *Pediococcus sp.* NWD 015 showed sensitivity to proteinase K (Table 2).

The inactivation of antimicrobial activity by proteinase K suggested that the substances evaluated in this study could be antimicrobial peptides or bacteriocins.

Treatment	Activity (AU/ml)	
	Enterococcus fecalis	*Staphylococcus aureus*
Control (blank)	400	600
Bakteriosin + *Proteinase* K	-	-
Proteinase K	-	-

Note : (-) not identified

(b). Activity of bacteriocins with heating treatment.

Table 4 showed that the bacteriocin activity from *Pediococcus sp.* NWD 015 still had activity after heating at 121°C for 15 min although the activity declined at least by 100 AU/ml and was stable after heating treatment at 100°C for 30 min (500 and 600 AU/ml). The stability of bacteriocin activity may be due to the cystein of contents that could stabilize the bacteriocin structure.

indicator	Activity after heating treatment (AU/ml)				
	Control*	100°C			121°C
		10 min	20 min	30 min	15 min
EF**	500	400	500	500	400
SA***	600	600	600	600	400

Note : * Control : without heat treatment, ** *Enterococcus faecalis*, *** *S. aureus*

(c). The stability of bacteriocins at different pH and storage condition.

The pH value was one of the important factors for bacteriocin production. In fact, bacteriocin was product by gene expression and significantly affected by the pH (Yang *et al.*, 1992). The stability of bacteriocin in different pH levels was shown in Table 5. Generally, bacteriocins from *Pediococcus sp.* NWD 015 were active in a wide range of pH from 2 up to 11 Bacteriocin was heated at 100°C before analysed it was indicated that the optimal activity at pH 6-7 The activity decreased by more than 50% at pH 8 to 11 compared pH 6 – 7. This result was similar with Izildinha *et al.* (2000) to the finding for produced nisin of ATCC 11454 by *Lactococcus sp.* which was stable at neutral and acid pH (2 – 6), partly active at pH 6 up to 10.

Bakteri indikator	Level pH							
	2,0	2,5	3,0	6,0	6,5*	7,0*	9,0	11,0
EF**	200	300	300	400	400	400	200	100
SA***	400	400	500	600	600	600	400	200

Note : * Control , ** *Enterococcus faecalis*, *** *S. aureus*

The bacteriocin remained stable during storage at 4°C for 10 days but decreased after storage at 20°C for 7 days.*Extraction of bacteriocin by Pediococcus sp. NWD 015*

with adsorbtion-desorbtion method

The bacteriocins produced by different species and strains of lactic acid bacteria have potential uses as biological food preservatives. In order to use them in the most effective ways, it will be important to obtain relatively large quantities of these peptides in a pure and concentrated form, and determine their physical and chemical characteristics as well as mode of inhibitory effect against food spoilage and food borne bacteria. For extraction, a novel purification method of Yang *et al.* (1992) was used. This method was based on pH dependancy of bacteriocins to adsorb and release from the cell surface of gram positive bacteria and produces dry preparations of bacteriocins with high potency and in a concentrated and pure form. As compared to ammonium sulphate precipitation of the bacteriocins from cell-free culture liquor which has low yield because of other proteins from the medium can also be precipitated and the yield is not very high; for extraction and purification of lactic acid bacteria, the total loss (in the novel method of Yang *et al*, 1991) is quite low and it is an ecomonical procedure to produce large quantities of bacteriocins from lactic acid bacteria to be used as food biopreservatives. Nevertheless in this research, the bacteriocin activity from *Pediococcus sp.* NWD 015 has decreased until 50%, but have high spesific activity. This suggested that since all in this process can be occured protease/proteolitic enzymes which can be reduce bacteriocin activity.

Analysis molecular weight bacteriocin produced by Pediococcus sp. NWD 015.

Bacteriocin from *Pediococcus sp.* NWD 015 was submitted to electrophoresis on 12% polyacrylamide gel in the presence of SDS. One gel containing the moleculer weight standard and sample (bacteriocin) was stained with brilliant blue G and the other gel containing sample treated with treatment sample buffer was used for identification of the bacteriocin band(s) by using growth inhibition of *S. aureus*. This result showed molecular weight of bacteriocin from *Pediococcus sp.* NWD 015 4.95 kDa.

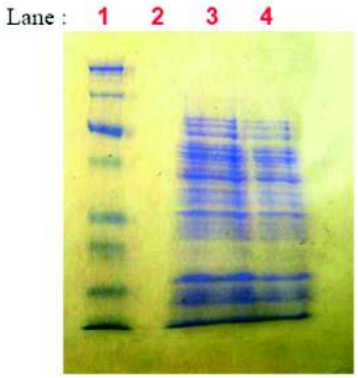

Figure 4. Coomassie-Blue stained SDS-PAGE gel I: lane 1 contain molecular weight standart (kDa) : a. 209; b. 124; c. 80; d. 49; e. 34; f. 28,9; g. 20,6; h. 7,1. Lane 2 contain a liquid TGE broth (control), lane 3 and 4 contain sample (extract) from Pediococcus sp. NWD 015 in different amounts; 10,0up and 15,0ul respecctively.

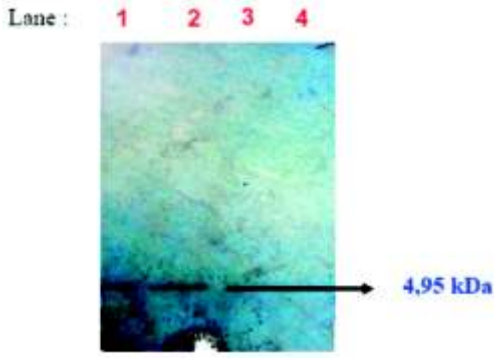

Figure 5. SDS-PAGE gel II: overlaid with indicator bacteria (S. aureus) to determine which band corresponds to the antimicrobial activity. Lane 1-3 contains sample (bacteriocin) from Pediococcus sp. NWD 015, showing the zone of growth inhibition corresponding the band molecular weight of 4,95 kDa. Other bandsdid not show antimicrobal activity. Lane 4 contains sample without treatment buffer and showed no growth inhibition zone.

Conclusions

The maximum bacteriocin production of *Pediococcus sp.* NWD 015 reached after

incubation of the cell 12 h at 37°C in TGE broth (600 AU / ml) and decreased after 96 h incubation, inactivated by Proteinase-K and active with heat treatment at 121°C for 15 min which its stable at 100°C for 20 – 30 min and over a wide range of pH (2 – 11). Bacteriocin from *Pediococcus sp.* NWD 015 was effective againts *Enterococcus faecalis, Stappyloocus aureus, Eschericia coli,* and *Listeria monocytogenes* (400, 600, 400, and 300 AU / ml), but not against *Salmonella thypimurium*.

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