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The antagonistic activity of lactic acid bacteria isolated from *peda*, an Indonesian traditional fermented fish

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Abstract. *Peda* is an Indonesian traditional fermented whole fish prepared by addition of salt prior to fermentation and drying process. Salt used to control the growth of the lactic acid bacteria for the fermentation process. The objectives of this study were isolating and characterize the potential lactic acid bacteria (LAB) from *peda* as culture starter candidate, particularly its activity against pathogenic bacteria. A total of five samples from five regions of East Java Province was collected and subjected to LAB isolation. Fifty-seven of 108 colonies that show clear zone in de Man, Rogosa and Sharpe (MRS) agar supplemented with 0.5% $CaCO_3$ were identified as LAB. Twenty-seven of the LAB isolates were exhibit inhibition against *Staphylococcus aureus* ATCC 6538 and *Pseudomonas aeruginosa* ATCC 27853. Isolate *Aerococcus* NJ-20 was exhibited strong inhibition against *S. aureus* ATCC 6538 (7.6 ± 1.35 mm inhibition zone) but was not produce bacteriocin. This finding suggests that the isolate *Aerococcus* NJ-20 can be applied as biopreservative culture starter on *peda* production. Further analysis on technological properties of isolates will be needed prior to application.

1. Introduction

Peda is an Indonesian traditional fermented fish product that contains 20-30 % (w/w) salt [1]. *Peda* is often produced from mackerel (*Rastrelliger* sp.) [2] with the addition of salt and put through a drying process to prolong the shelf life of the fish. Salt in fermented fish inhibits the growth of spoilage and pathogenic bacteria, as well as maintaining lactic acid bacteria for the fermentation process [3].

Lactic acid bacteria (LAB) is Generally Recognized as Safe (GRAS) by WHO and plays an important role in the process of fermentation of food such as inhibiting spoilage bacteria and producing the flavour, aroma, and texture of fermented food [4,5]. LAB may be isolated from fermented fish such as *plasom*, *bekasam*, fish sauce, and *peda* [6,7,8,9].

The antagonistic activity of LAB is due to antimicrobial compounds produced by the bacteria such as lactic acid, hydrogen peroxide (H_2O_2), diacetyl, reuterin, and bacteriocin [10]. Lactic acid can disrupt the outer membrane of bacteria that causes lysis, whereas bacteriocins are antimicrobial proteins or peptides synthesized by ribosomes [11] that initiate pore formation on the bacterial

membrane. Bacteriocin has bactericidal or bacteriostatic properties against spoilage and pathogens bacteria and is used as a biopreservative in food products [12,13].

The antagonistic activity of LAB was isolated from various fermented fish such as *bekasam* [14]. The different regions of production and methods performed during the production of *peda* may lead to differences in LAB isolation as well as their antagonistic activity against foodborne pathogens. The objective of this study was to isolate and analyze the antagonistic activity of LAB from *peda* as a culture starter of *peda* production.

2. Methodology

2.1 Samples collection

Sample collection was conducted by the method of Cho [15]. Mackerel *Peda* (*Rastrelliger* sp.) samples were collected from five regions of East Java (Surabaya, Sidoarjo, Gresik, Kediri, and Nganjuk). All samples were wrapped in sterile plastic bags, stored in an insulated cool box (4^{o}C) and transported to the laboratory within 5-8 hours.

2.2 Isolation of lactic acid bacteria (LAB)

The isolation of lactic acid bacteria was done according to Angmo et al [16] and Pranomo et al [17]. A total of 10 g sample of mackerel *peda* was homogenized with 90 ml of normal saline (0.85 % w/v) and diluted. One mL of the diluted sample was spread on de Man, Rogosa and Sharpe (MRS, Merck, Germany) Agar supplemented with 0.5 % $CaCO_3$, and then incubated at 30^{o}C for 48 hours. Individual colonies were picked and streaked on MRS supplemented with $CaCO_3$ and incubated at 30^{o}C for 24 hours. Purified cultures were tested for catalase and Gram staining. All catalase negative and Gram-positive strains were stored in MRS broth with the addition of sterile glycerol (1:1 v/v) at -20^{o}C.

2.3 Antagonistic activity of LAB

The isolates of LAB were cultured on de Man, Rogosa and Sharpe (MRS, Merck, Germany) broth and incubated at 30°C for 24 hours twice prior to the antagonistic test. The indicator bacteria (*Staphylococcus aureus* ATCC 6538 and *Pseudomonas aeruginosa* ATCC 27853), which represented Gram-positive and Gram-negative bacteria respectively, were cultured on Trypticase Soya Broth (TSB, Oxoid, UK) and incubated at 30°C for 24 hours. Each indicator bacteria (10^7 CFU/ml) was swabbed on a Mueler Hinton Agar (MHA, Oxoid, UK) medium using a sterile cotton-swab. LAB culture was applied at 20 µl into a sterilized paper disk. The paper disk was then placed on the surface of the MHA medium that had been swabbed with indicator bacteria and incubated at 30^{o}C for 24 hours. LAB isolates that formed the inhibited zone (≥ 6 mm) were selected for the bacteriocin production test [18].

2.4 Bacteriocin-producing test

The LAB isolates which inhibited indicator bacteria were cultured on de Man, Rogosa and Sharpe (MRS, Merck, Germany) broth medium and incubated at 30^{o}C for 24 hours. The culture was then centrifuged (Hettich Rotanta 420 R, Germany) at 4600 rpm at 4°C for 10 minutes, then the pH adjusted to 7.0 with 1 N NaOH. The neutralized-cell free supernatant is heated at 80°C for 10 minutes to eliminate hydrogen peroxide and kill the cells. The neutralized-cell free supernatant was filtered with 0.22 µm millipore filter. The antimicrobial activity of the supernatant was confirmed by the Agar Well Diffusion Assay (Pringsulaka et al. 2012) against the indicator bacteria *S. aureus* ATCC 6538. MHA (Merck, Germany) soft agar (0.75 % agar w/v) which was inoculated with 1 ml of indicator bacteria 10^7 CFU/ml. A 50 µL cell free neutralized supernatant was loaded to each well and incubated at 30 °C for 24 hours. The isolate that exhibited antimicrobial activity in the AWD test was then characterized by the nature of its activity by adding proteinase K. The neutralized-cell free supernatant that lost activity by the addition of proteinase K indicates bacteriocin production [30]. Briefly, the proteinase K of 200 µg (w/v) was dissolved in a 1 ml phosphate buffer solution and then added to the neutralized-cell free supernatant. The neutralized-cell free supernatant was incubated at 30 °C for 2

hours. The activity of the proteinase K enzyme was stopped by heating it at the temperature of 100 ^{o}C for 30 minutes. The neutralized-cell free supernatant was tested against *S. aureus* ATCC 6538 with Agar Well Diffusion Assay.

2.5 Identifitication of LAB

Identification was performed to find the genus of LAB that showed high antagonistic activity. The characterization included performing tests on gas production (fermentation type), pH effect, temperature influence, and salinity and comparing them to the manual identification of LAB.

2.6 Data analysis

All measurements of antagonistic activity were performed in triplicate. The data was expressed in the mean and standard deviation of the three experiments.

3. Results and Discussion

3.1 Isolation of LAB

A total of 108 colonies that exhibited a clear zone on de Man, Rogosa and Sharpe agar supplemented with $CaCO_3$ 0.5% was purified in MRS. Among 108 isolates, only 57 isolates (52.7 %) were included as lactic acid bacteria due to it being Gram-positive, rods and cocci-shaped, and negative catalase. LAB shows a clear zone on MRS supplemented with $CaCO_3$ due to the production of organic acid (such as lactic acid, propionic acid, and/or acetic acid) [5] that dilutes the $CaCO_3$ in the medium, then the organic acids can react with $CaCO_3$ forming Ca-lactate and forms a clear zone [6,19]. LAB is a Gram-positive, non-spore forming, rod or cocci-shaped, catalase negative, and non-motile bacteria. In addition, from the data of Gram staining (*data not shown*), 12 and 45 isolates out of 57 isolates LAB were rod and cocci-shaped respectively.

The isolating rate of lactic acid bacteria was correlated with various factors such as the location, method, and source of LAB. Angmo et al [16] was an isolated LAB from a fermented beverage of Ladakh and found 46 LAB isolates from dried cottage cheese and beverage. Pringsulaka et al [20] isolated 152 LAB from 93 samples of Thai fermented meat and fish product. Furthermore, 160 LAB strains were isolated from the digestive tracts of marine fish and evaluated of potential use as probiotics by Buntin et al [21]. Additionally, there are 51 strains of LAB isolated from the intestinal tract of Kuruma shrimp (*Marsupenaeus japonicus*) and the selected strain for probiotic [22].

The total bacterial count on the samples is well known to be correlated with the source of the raw materials. In the case of fermented fish, the main source of LAB is the gastrointestinal tract of the fish. Ringø [23] reported a minor number of LAB from the Arctic charr, *Salvelinus alpines*, approximately 10 % from the gastrointestinal microflora of *Arctic charr*, *Salvelinus alpines*. In contrary, Buntin at al [21] reported the high number of LAB from warm-water or tropical fish, approximately 72.5 % of the total isolated LAB. In this study, the low number of LAB (52.7 %) may be due to the high concentration of salt employed to produce the *peda* fermented fish. The addition of salt during *peda* fermentation reduces the water activity of its food matrix. This condition inhibits the growth of Gram-negative bacteria and promotes the growth of Gram-positive bacteria such as LAB. Therefore LAB plays a central role in fish fermentation along with the addition of salt [24]. Salt is used to control the growth of desirable bacteria such as lactic acid bacteria [25].

3.2 Antagonistic activity of isolated LAB

Among 57 isolates, 27 isolates exhibited inhibition activity (Table 1) and only one isolate exhibited strong activity against *S. aureus* ATCC 6538, namely NJ-20 with the inhibition zone of 7.6 ± 1.35 mm. The inhibition of pathogenic bacteria by LAB is due to the acidification of the medium. Organic acids are substances produced by LAB that inhibit Gram-negative bacteria such as *Yersinia enterocolitica*, *Salmonella* sp., *Escherichia coli*, *Pseudomonas* sp. [16,10,6]. The inhibition of pathogenic bacteria may be due to more than one mechanism, which includes suppression of growth, production of organic acids (such as lactic acid), and active compounds (bacteriocins) [24,26].

Table 1. Antagonistic acticity of isolated lactic acid bacteria from Peda against *S. aureus* ATCC 6538 and *P. aeruginosa* ATCC 27853.

Isolates	Inhibition Zone (mm)[a]	
	S. aureus ATCC 6538 [a]	*P. aeruginosa* ATCC 27853 [a]
NJ-15	2.9 ± 0.46[a]	0 ± 0
NJ-17	2.2 ± 1.46	0 ± 0
NJ-18	2.6 ± 0.75	0 ± 0
NJ-20	7.6 ± 1.35	0 ± 0
NJ-21	4.7 ± 1.38	1.1 ± 0
KD-1	2.3 ± 1.25	0 ± 0
KD-3	2.3 ± 1.35	0 ± 0
KD-6	1.3 ± 0.40	0.6 ± 0.55
GR-2	0 ± 0	4.5 ± 0.51
GR-3	0 ± 0	1.8 ± 0
GR-8	0 ± 0	3.5 ± 0
GR-10	0.9 ± 0	0 ± 0
GR-11	0 ± 0	1.5 ± 0
GR-13	0 ± 0	2.3 ± 0.2
GR-14	0.9 ± 0	0.7 ± 0.75
SD-18	0 ± 0	0.9 ± 0
SD-19	0 ± 0	2.2 ± 0
SD-20	0 ± 0	0.8 ± 0
SD-21	0 ± 0	1.1 ± 0
SD-22	0 ± 0	1.2 ± 0
SD-24	0 ± 0	2.7 ± 0
SD-25	0 ± 0	0.7 ± 0
SD-28	3.1 ± 0.36	0 ± 0
SB-1	1.7 ± 0	0 ± 0
SB-4	2.8 ± 0.83	0 ± 0
SB-19	0 ± 0	1.7 ± 0
SB-20	0 ± 0	3.4 ± 0.87

[a]Mean ± SD

The LAB NJ-20 isolate was then selected for the detection of bacteriocin producing activity against *S. aureus* ATCC 6538 due to its inhibitory zone of ≥ 6 mm [18]. Gram-negative is susceptible to organic acids because the cytoplasmic membrane of Gram-negative bacteria is damaged by organic acids [27]. In this study, the strong activity of LAB NJ-20 isolate against Gram-positive bacteria (*S. aureus* ATCC 6538) indicates the potential for bacteriocin production.

3.3 Bacteriocin-producing test of isolate LAB NJ-20

The bacteriocin-producing test employed an Agar Well Diffusion Assay (AWDA) of LAB NJ-20 isolate which was obtained from *peda*. However, the neutralized-cell free supernatant of the NJ-20 did not exhibit a clear zone around the well against *S. aureus* ATCC 6538. This finding indicates that the neutralized-cell free supernatant did not contain an antimicrobial substance such as bacteriocin. Bacteriocin is an antimicrobial peptide or protein that is heat-stable, resistant to pH, sensitive to protease and inactivated by the digestive enzyme [12].

The incubation temperature and pH play an important role in the production of bacteriocin, because an optimum temperature and pH are required for bacterial growth so as to produce bacteriocin. The optimal range of temperature and pH to produce bacteriocins depend on the strains of the producer bacteria [4,28]. The optimal temperature and pH stimulate LAB to produce bacteriocin at the beginning of the exponential phase. According to Ponce *et al.* [29] bacteria produces bacteriocin exponentially in the initial phase and is inactivated at the stationary phase. The phase of lactic acid bacteria in producing bacteriocin varies depending on the strains of the producer bacteria [29]. The long incubation time resulted in the bacteriocin being degraded by a bacterial secreted enzyme [30].

However, LAB is known to produce a wide range of antimicrobial compounds (organic acids, bacteriocin, diacetyl and hydrogen peroxide) [10,24,26] that can still be applied as biopreservation. LAB had been granted Generally Recognized As Safe (GRAS) status by the US Food and Drug Administration [24]. Meat-borne LAB generally inhibits other LAB species as well as Gram-positive bacteria such as *Staphylococcus aureus*, *Listeria monocytogenes*, *Clostridium perfringens*, *Clostridium botulinum*, *Enterococcus faecalis*, and *Bacillus* spp. due to the formation of pores in the cytoplasmic membrane of sensitive strains [31].

Table 2. Identfication of NJ-20 isolate.

Identification	Isolate LAB NJ-20	*Aerococcus*
Shape	Cocci	Cocci
Shape tetrad	+	+
Gram-positive	+	+
Catalase	-	-
CO_2 from glucose	-	-
Growth at 10 ^oC	+	+
Growth at 45 ^oC	-	-
Growth in NaCl 6.5 %	+	+
Growth in NaCl 18 %	-	-
Growth at pH 4.4	-	-
Growth at pH 9.6	+	+

3.4 Identification of LAB NJ-20

The genus identification based on morphology and physiology tests indicate that NJ-20 belong to the *Aerococcus* genus. The results of identification of NJ 20 isolates are shown in table 2. These results were different with the previous study on the identification of LAB from *peda* which found Lactic acid bacteria isolated from *peda* consist of *Lactobacillus plantarum*, *L. curvatus*, *L. murinus*, *Streptococcus thermophilus*. The difference may be due to the different sampling locations as well as the raw materials used. *Aerococcus* is included in the *Lactobacillales* order which is closely related to the *Streptococcus*, *Lactococcus*, *Carnobacterium*, *Vagococcus* and *Enterococcus* species. The *Aerococcus* species lives in a wide range of environments including air, vegetation, meat-curing brines, soil, and marine sources [32].

4. Conclusion

The antagonistic activity of lactic acid bacteria isolated from *peda*, a traditional fermented fish from Indonesia, suggested that 27 isolates exhibited an antagonistic effect against *S. aureus* ATCC 6538 and/ or *P. aeruginosa* ATCC 27853. Strong inhibition to *S. aureus* ATCC 6538 was performed by the LAB NJ-20 isolate. The inhibitory activity of *Aerococcus* NJ-20 was not due to the production of bacteriocin. This finding suggests that Aerococcus NJ-20 may be applied as a biopreservation ingredient for fishery products as well as a culture starter of *peda* fermentation. Nevertheless, further studies are needed to evaluate the enzyme activity, safety, growth of isolates on the fermentation process and biogenic amine production prior to applications.

5. References

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