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ANIMAL SCIENCE

Characterization of lactic acid bacteria isolated from an Indonesian fermented fish (bekasam) and their antimicrobial activity against pathogenic bacteria

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Abstract

Bekasam is an Indonesian fermented fish product that tastes sour and mostly contains lactic acid bacteria (LAB). The aim of this study is to obtain and characterize LAB isolates from bekasam and to study their potency in inhibiting the growth of pathogenic bacteria, i.e. Escherichia coli, *Salmonella typhimurium* ATCC 14028, *Bacillus cereus, Staphylococcus aureus* and *Listeria monocytogenes*. LAB were isolated from bekasam using media of MRSA supplemented with $CaCO_3$ 0.5%. Incubation was at 37°C for 48 hours. The pure cultures were verified as LAB based on morphological and biochemical characteristics. Eight bekasam samples showed that the total average of LAB were 1.4 x 10^8 to 9.0 x 10^8 CFU/mL. Seventy four isolates were successfully isolated. It was found that 62 isolates (84%) belonged to LAB, and 23 isolates of them could inhibit the growth of the five pathogenic bacteria. The highest inhibition zone was accounted for S. aureus. However, neutralized supernatant of the LAB culture did not inhibit the growth of the pathogenic bacteria. These results indicate that the LAB inhibition to the pathogenic bacteria was due to the organic acid, and that perhaps the main factor in the bekasam preservation by LAB.

Key words: Antimicrobial, Bekasam, Fermented fish, Inhibition index, Lactic acid bacteria

Introduction

Bekasam is an Indonesian fermented fish product with a sour taste and it is a popular food in Central Java, South Sumatra and South Kalimantan. Generally the production of bekasam involves a spontaneous, fermentation process of freshwater fish, supplemented by salt, and rice or fermented cassava (Murtini et al., 1997).

Lactic acid bacteria LAB is the dominant microorganism found in fermented fish. In som-fak, rapid growth of LAB cousing pH to decrease below 4.5 in two days is essential to prevent spoilage and to ensure safety of the product (Ostergaard et al., 1998). LAB has an important role in food fermentation that caused changes in taste, smell and texture with improved preservation of the product (Hugas, 1998). This preservation process inhibits the growth of spoiled and pathogenic bacteria. However the salt too had ability to inhibit the growth of spoiling and pathogenic bacteria, because of antibacterial activity. The LAB produces some metabolites i.e. organic acid (lactic acid and acetate), hydrogen peroxide, diacetil and bacteriosin (Ross et al., 2002; Diop et al., 2007; Galves, 2007).

LAB was reported in the fermented fish product from Thailand such as pla-ra, pla-chom, plaa-som and som-fak. The dominant bacteria on fermented product of som-fak, plaa-som, pla-ra and pla-chom were LAB i.e. *Lactococcus lactis* subsp. lactis, *Leuconostoc citreum*, *Lactobacillus paracasei* subsp. paracasei, *Weisella confuse*, *L. plantarum*, *L. pentosus, Pediococcus pentosaceus*, *Lactobacellus alimentarius/farciminis, Lactococcus garvia, Lactobacillus acidipicis* sp. nov and *Weissella thailandensis* sp nov (Paludan-Muller et al., 1999; Tanasupawat et al., 2000; Paludan-Muller et al., 2002). *Weissella cibaria* 110 (plaa-som) produced bacteriosin known as weissellicin 110 (Sriounnual

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et al., 2007), and plantaricin w produced by *Lactobacillus plantarum* PMU33 (som-fak) (Noonpakdee et al., 2009). However, there is limited information on the microbiological aspects of bekasam fermentation due to the fact that its production is on a small or house-hold scale. Rahayu (2010) stated that most research in Indonesia related to LAB is mostly in the area of exploration for the potency of these bacteria in fermented foods, biopreservative and probiotics. Therefore this study describes the characteristics of LAB isolated from bekasam and their inhibitory action on the growth of pathogenic bacteria.

Materials and Methods

Isolation and Selection of LAB

Bekasam was collected from local small scale processors in Panganjang, Indramayu residence (West Java), Indralaya, Ogan Ilir Residence (South Sumatera) Cengal and Kayu Agung, Ogan Komiring Ilir Residence (South Sumatera). Each sample was measured for its pH, NaCl and lactic acid contents using titration method. The number of cell was measured using plating method on Plate count Agar (PCA) for the total aerobic bacteria and de Man Rogosa Sharper Agar (MRSA) supplemented with $CaCO_3$ 0.5% for total of LAB (Veljovic et al., 2007). LAB were isolated from the plates of MRSA. As many as 10-20 of colonies were isolated using scratched plating method. Incubation was at 37°C for 48 hours (Tanasupawat et al., 1998). The pure culture isolate was verified as LAB using gram staining, spore staining, motility, catalase test, gas production from glucose (Palludan-Muller et al., 2002; Kopermsub et al., 2006; dan Veljovik et al., 2007).

Antimicrobial activity test

Antimicrobial activity of LAB was determined using double layer method against *E. coli, S. typhimurium* ATCC 14028, *B. cereus, S. aureus,* and *L. monocytogenes* (Nurhasanah, 2004). As much as 50 µL of the idicator bacteria (10^8 CFU/mL) was suspended in 50 mL Nutrient Agar (NA) consisted of 0.75% agar and then poured 10 mL into the surface of lactid acid isolate on MRSA media. All the culture was incubated at 37°C for 24 hours. The bacteria having an ability to produce antimicrobial compound showed clear zone (inhibition zone) around the colony. The inhibition index was calculated using the following formula:
Inhibition index=(inhibition zone diameters (mm)-colony diameters (mm))/(colony diameters (mm)).

Isolates of lactic acid bacteria showed antagonistic activity against indicator bacteria was examined for their antimicrobial compound using agar well diffusion method.

Culture supernatant was obtained from an overnight culture by centrifugation at 10.000 rpm for 10 min at 4°C.The supernatant was neutralized (at pH 7.0±0.2) with 1N NaOH, and sterilized by filtering with acrodisc (pore size 0.22 µm). The antimicrobial activity of neutral supernatant was carried out by using agar well diffusion methods. A volume of 20 µL culture of the indicator bacteria was inoculated to 20 mL of liquid Molten Muller Hinton agar, and after it solidified the cultures were incubated overnight. The culture was bored using cork bore (5 mm in diameter). As much as 70 µL of cell-free supernatant (CFS) was dispensed into the wells. All plates were incubated overnight at 37°C. Inhibition zone was determined by measuring clear area surrounding agar well with modifications Diop et al. (2007).

Results and Discussion

LAB from Bekasam

Eight samples of bekasam taken from 4 locations contained 2.34% - 7.28% and 1.13% - 2.50% of total salt and lactid acid, respectively (Table 1). Before fermentation, bekasam consisted of salt 15% - 25% and carbohydrate source from rice 30% - 50 %. LAB total in bekasam was $1.4x10^8$-$9.0x10^8$ CFU/mL. Lactic acid produced by LAB was decreased pH up to 3.60 – 5.30. Adding of salt could inhibit growth of spoiled bacteria. This indication was shown from the data of aerobic microbes that was smaller than the total of LAB (Table 1).

Seventy four isolates of acid producing bacteria were examined for their morphological and biochemical characteristics i.e. catalase production, motility and gas production from glucose. Lactic acid bacteria generally were defined as a group of lactic acid producer, which have characteristics of low G+C, non-spore forming, gram-positive rod and cocci, fermentative, negative catalase, anaerob facultative, non-motile and tolerant against acid (Hutkins, 2006). The result of morphological and biochemical characteristics of LAB from bekasam was shown on Table 2.

Based on the morphological characteristic with gram's staining, the percentage of rod shaped cells to sphere shape cells was determined. Among the 74 isolates, 54% were gram-positive with rod shaped cells (bacilli) and 46% gram-positive with sphere shape cells (cocci). And 1% was spore forming bacteria and 99% non-spore forming bacteria. All isolates were non-motile. Eleven isolates performed positive reaction for the catalase

test. Based on gas production from glucose fermentation test, 69 isolates had no gas formation from glucose fermentation. Based on morphological and chemical characteristics it was concluded that 62 isolates belonged to LAB group.

In a study that investigated milk fish bekasam, Chandra et al. (2007) stated that among the five acid producing isolates isolated from bekasam, four were Gram-positive cocci and one was gram-positive bacilli. All isolates were no spore and non motil. This research was also obtained that LAB isolates were isolated from milk fish bekasam (BI.8 sample) was dominated by gram-positive cocci, and all isolates were also no spore and non motil (Table 2).

Table 1. Bekasam characteristics and their chemical and microbiological parameters.

No	Sample	Location	Type of fish	Length of Fermentation (day)	pH	Salt concentration (%)	Total of lactic acid (%)	Total of aerobic microbe (CFU/ml)	Total of LAB (CFU/ml)	Isolates obtained
1	BP.4	Indralaya, Ogan Ilir residence (South Sumatera)	Pearl gourami	4	4.60	2.34	2.41	$1.7x10^8$	$4.0x10^8$	14
2	BP.8		Pearl gourami	8	3.62	3.45	241	$1.2x10^8$	$3.2x10^8$	4
3	SI.7		Rasbora	7	3.71	7.28	2.41	$6.6x10^6$	$1.4x10^8$	10
4	SK.7	Kayu Agung, Ogan Komiring Ilir residence (South Sumatera)	Rasbora	7	3.60	4.62	1.43	$1.2x10^8$	$5.0x10^8$	7
5	NS.4	Desa Sungai Pasir, Distric of Cengal, Ogan Komiring Ilir residence (South Sumatera)	Nile tilapia	4	5.30	nd	1.13	$4.2x10^8$	$9.0x10^8$	7
6	SS.8		Pearl gourami	8	4.45	nd	2.50	$5.6x10^7$	$4.8x10^8$	12
7	PS.8		Cat fish	8	4.23	nd	1.67	$4.3x10^7$	$4.7x10^8$	5
8	BI.8	Panganjang, Indramayu residence (West Java)	Milk fish	8	4.09	4,01	2.20	$4.8x10^7$	$2.7x10^8$	15
Total										74

nd= not determined

Table 2. Number of lactic acid bacteria isolated from bekasam with their morphological and biochemical characteristics.

No	Sample code	Isolates code	Number isolates	Number of isolates with Cell morphology			Biochemical properties				Number of LAB isolates
				cocci	bacilli	Gram-positive	No spore	Negative catalase	Non motile	Homo fermentative	
1	BP.4	BP (1-20)	14	7	7	14	14	13	14	13	13
2	BP.8	BP(21-30)	4	0	4	4	3	4	4	4	3
3	SI.7	SI(1-15)	10	9	1	7	10	4	10	10	4
4	SK.7	SK(1-20)	7	3	4	7	7	6	7	7	6
5	NS.4	NS(1-17)	7	0	7	7	7	7	7	7	7
6	SS.8	SS(1-17)	12	2	10	12	12	12	12	9	12
7	PS.8	PS(1-17)	5	0	5	5	5	5	5	5	5
8	BI.8	BI(1-20)	15	13	2	15	15	12	15	14	12
		Total	74	34	40	74	73	63	74	69	62

In a study that investigated plaa-som, a similar fermented fish product likes bekasam. Kopermsub et al. (2006) stated that among the ninety acid producing bacterial isolates isolated from plaa-som, 79% were gram-positive rod shaped cells (bacilli) and 21% gram-positive sphere shape cells (cocci). From 90 isolates, eighty isolates were confirmed as belonging to the LAB group. Three genera were identified as *Lactobacillus* spp., *Pediococcus* spp., and *Aerococcus* spp. at 79%, 18% and 3%.

Yahya et al. (1997) reported that LAB isolated during bekasam fermentation from Nile tilapia (*Oreochromis mossambicus*) were *Leuconostoc mesenteroides* which was isolated from sample of 1 – 7 days fermentation. *Lactobacillus acidophilus* from day 5 to 7, *L. plantarum* and *L. fermentans* on the first day, *L. buchneri, L. reuteri* on the third day, *Pediococcus pentacaseus, L. bifermentans, L. tolerans, Pediococcus acidilactici, L. bulgaricus*, Leu. dextranicum are isolated on the seventh day. There are four isolates that have antimicrobial activity against *Staphylococcus aureus* FNCC 0047; they are *Lactobacillus plantarum*-IB2, L. fermentum-IB5, *L. acidophilus* IIIB5 and *Pediococcus acidilactici*-IVB2.

Gram-positive rod shaped cells (bacilli) were generally the predominant bacteria in all samples. While gram-positive sphere shaped cells (cocci) were more dominant in bekasam of rasbora (*Rasbora* sp.) from Indralaya and milk fish (*Chanos chanos*) bekasam from Panganjang. This difference might be caused by the type of raw material utilised. Bekasam processing still uses spontaneous fermentation process so the bacteria dominating in this fish fermentation process is indigenous bacteria that come from each of the raw material mainly the fish.

Some researchers reported that LAB is part of native microbiota of aquatic animals (Ringo, 2004). Itoi et al. (2008) reported that halotolerant strains of *Lactococcus lactis* subsp lactis isolated from the intestinal tract of the pufferfish *Takifugu niphobles* caught in Shimoda, Shizuoka, Japan. Nair and Surendran (2005) isolated lactic acid bacteria from various samples of fresh and frozen fish and prawn. Thirteen species of *Lactobacillus* were identified among the 64% isolates. Among them, *L. plantarum* was the dominant species. The remaining 36% isolates of *Lactobacillus* could not be assigned to any species with the available taxonomic schemes.

According to Kopermsub et al. (2006) fermented food characteristics varied with raw material and additional material used that caused the microbial diversity in the food. Palludan-Muller et al. (1999) reported that LAB bacteria isolated from fish, rice, garlic and banana leaf and during som-fak fermentation process. *Lactococcus lactis* subsp. lactis and *Leuconostoc citreum* were specifically associated with fish fillet and minced fish, *Lactobacillus paracasei* subsp. paracasei with boiled rice and *Weisella confusa* with garlic mix and banana leaves. In addition, *Lactobacillus plantarum*, *Lactobacillus pentosus* and *Pediococcus pentosaceus* were isolated from raw materials. A succession of aciduric, homofermentative lactobacillus species, dominated by *L. plantarum/pentosus*, was found during fermentation.

This study showed that 69 isolates consisted of homofermentative characteristics. It showed that these isolates had the ability to change more than 90% of sugar substrates to become lactic acid. This is different from heterofermentative bacteria that produce less than 50% of lactic acid and 50% acetate acid, ethanol and carbon dioxide. According to Rose et al. (2002) the group of homofermentative bacteria consists of *Lactococcus, Pediococcus, Enterococcus, Streptococcus* and some *Lactobacillus* use Embden-Meyerhof-Parnas pathway to change glucose to lactic acid.

Antimicrobial activity of LAB isolates

As many as 62 LAB isolates from bekasam were tested for their antimicrobial activity against five indicator bacteria associated with foodborne deseases. Figure 1 shows that five of LAB isolates had antimicrobial activity against five indicator bacteria.

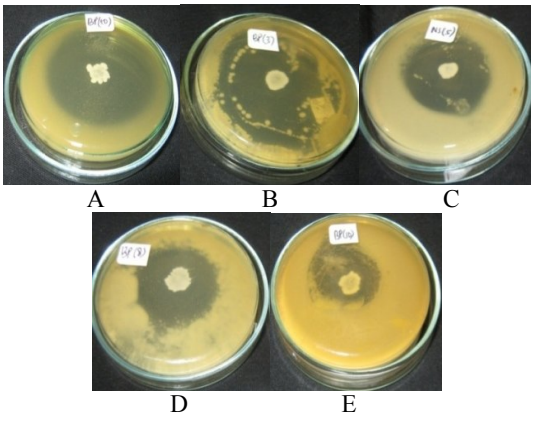

Figure 1. Antimicrobial activity of LAB isolates against indicator bacteria of *E. coli* (A), *S. typhimurium* (B), *L. monocytogenes* (C), *B. cereus* (D), dan *S. aureus* (E) with double layer method.

Table 3. The range of inhibition zone and Inhibition index on each indicator bacteria.

No.	Indicator bacteria	Number of isolates that inhibit	Inhibition zone (mm)	Inhibition index
1	*L. monocytogenes*	56 (90%)	2 -29	0.3 – 5.4
2	*S. typhimurium*	49 (79%)	3 -38	0.4 – 5.4
3	*E. coli*	45 (73%)	4 -32	0.3 – 4.0
4	*B. cereus*	44 (71%)	2 - 33	0.3 – 4.7
5	*S. aureus*	41 (66%)	2 - 44	0.2 – 6.1

There were 23 (38%) isolates that inhibited the five indicator bacteria. And 56 (90%) isolates of LAB produced inhibition zone against *L. monocytogenus*, while the LAB isolates that inhibit growth of *S. typhimurium, E.coli, B. cereus* and *S. aureus* were 79%; 73%; 71% and 66% respectively. The highest range of inhibition zone and inhibition index was on *S. aureus* (Table 3).

All isolates exhibited inhibition zones against the indicator bacteria which is indicative of their antimicrobial activity. Each isolate had different ability to inhibit growth of the five indicator bacteria. The inhibition activity of various antimicrobial substances against several species of pathogen bacteria was different. The antimicrobial activity of LAB was mainly caused by organic acid produced from glucose metabolism. At this study, the concentration of lactic acid for all samples was 1.13 – 2.50% with pH up to 3.60 – 5.30. LAB can produce some metabolites like organic acid (lactic and acetate acid), hydrogen peroxide, diacetil and bacteriosin (Ross et al., 2002; Diop et al., 2007; Galves, 2007).

All the neutralized cell-free supernatant of 53 LAB isolates did not show the inhibition zones against the five indicator bacteria. While non-neutralized cell-free supernatant showed inhibition zone about 1–10 mm. This indicates that inhibition activity of LAB isolates was caused by the lower pH of supernatant due to organic acid content. The supernatant pH after incubating for 24 hours from 53 LAB isolates was in range of 3.5 to 5.7. This indicated that the antimicrobial activity of LAB isolates from bekasam was caused by organic acid. Palludan-Muller et al. (2002) reported that the primary role of LAB is to ferment the available carbohydrates thereby decreasing in pH. It is important to inhibit pathogenic and spoilage bacteria and guaranty of product safety. The organic acids produced (mainly lactic acid) is the main preservative factor in bekasam fermented fish products.

The positive control of lactic acid solution with a pH of 4, 5 and 6 showed that at pH 5 and 6 did not show inhibitory zone against the five indicator bacteria, whereas at pH 4 showed inhibitory zones in range of 3-7 mm. According to Allokami et al. (2000), antimicrobial activity of lactic acid was shown at concentration of 5mM or at pH 4. This acid causes prominent permeabilization on the outer membrane of *Escherichia coli* O157: H7, *Pseudomonas aeruginosa*, and *Salmonella enterica serovar typhimurium.*

Conclusions

Sixty-two isolates obtained from bekasam were member of the LAB group. And 90% of LAB isolates inhibited growth of *L. monocytogenes*. And the percentage of LAB isolate that inhibited the growth of *S. typhimurium, E. coli, B. cereus* and *S. aureus* were 79%, 73%, 71% and 66%, respectively. The highest inhibition zone and inhibition index was on *S. aureus*. However, culture supernatant of the LAB isolates did not produce inhibition zone at neutral pH. This result indicates that inhibition activity of LAB from Indonesian bekasam was from organic acids, and they are probably the main preservative factor in the bekasam. The antibacterial compounds produced by LAB or its LAB obtained from bekasam may be used to combat the growth of pathogenic microorganisms in the bekasam fermentation processing. They could give a beneficial effect to fermented product, ie more stable product and metabolites which are produced during fermentation will improve flavor, aroma and texture of the product. Base on this results, improvement of bekasam fermentation could carry out, using LAB isolates as a starter culture. This is possible utilization for large scale production.

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