Organic acid produced by lactic acid bacteria from bekasam as food biopreservatives

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Abstract. Bekasam is one of the Indonesian fish fermented products that contain lactic acid bacteria (LAB). Previous research has obtained four LAB strains which are considered as bacteriocin producers, have antibacterial activity against pathogenic bacteria from food. In addition to a bacteriocins, LAB produce another compounds, such as organic acids. This study aimed to determine the produced organic acids and their antibacterial activity during LAB growth against of Lactobacillus monocytogenes, Staphylococcus typhimurium, Escherichia coli, Bacillus cereus and Staphylococcus aureus, to determine the organic acid contents using HPLC and to identify LAB isolates using molecular method based on 16S rDNA sequences. The four LAB strains (BP (3), BP (20), BI (3) and SK (5)) were grown in MRS broth medium, then incubated at 37 °C for 48 hours and every 4 hours incubation was tested of titratable acid total (TAT), pH, LAB growth and antibacterial activity. The result showed that the exponential phase of bacteria growth occurred at 16-20 hours incubation and at the end of the exponential phase produced the highest antibacterial activity in the four LAB strains. The highest growth, TAT and antibacterial activity produced by the SK (5) strain. The highest organic acids content were lactic acid in the SK(5) strain and acetic acid in the BI(3) strain. Identification result indicated that BI(3), BP(3), and BP(20) strains were Pediococcus pentosaceus IE 3 with similarity of 98%, 97%, and 98%, respectively. While SK(5) strain showed 93% similarity to Lactobacillus plantarum subsp. plantarum NC 8. The four LAB strains produced organic acids and had antibacterial activity, so they can be developed as food biopreservatives.

Keywords: antibacterial activity; Lactobacillus plantarum; Pediococcus pentosaceus; the organic acid content.

1. Introduction
Bekasam is indigenous Indonesian fish fermented product that tastes sour, so it contains a lot of lactic acid bacteria. This product is widely known in the regions of Central Java, South Sumatera and South Kalimantan. The fermentation products as well as beneficial bacteria are generally selected in this process to control spoilage and render pathogen inactive. The special interest organism or central
organism used for this purpose is lactic acid bacteria (LAB) and their metabolites. They are capable to exhibit antimicrobial properties and helpful in imparting unique flavour and texture to the food products. The major compounds produced by LAB are bacteriocin, organic acids and hydrogen peroxide which it can used as biopreservative. Bio-preservation is a technique of food preservation in which antimicrobial potential of naturally occurring organisms and their metabolites are exploited [1-3]. Ray and Joshi also reviewed about fermented foods: past, present and future, they stated that the most important bacteria in the fermentation of foods are the Lactobacillaceae, which have the ability to produce lactic acids from carbohydrates [2, 5].

Organic acids are the most commonly used preservatives in food, have generally recognized as safe (GRAS) status, and have a broad spectrum of antibacterial agents. Organic acids are effective for preserving food because in addition to antibacterial activity, they also act as acidulants. Lactic acid is one of organic acid that is widely used in broad industrial applications. Lactic acid is also a hydroxy acid which is classified as GRAS by the Food Drug Administration (FDA) and is very often used in food as an acid enhancer, flavor ingredient, pH buffer material, and preservative [4, 6].

Lactic acid is the end product of carbohydrate fermentation by LAB. However, it can be produced commercially by chemical synthesis and fermentation. Chemical synthesis produces a mixture of two isomers while during fermentation a purely optical form of lactic acid is produced. About 90% of the world's total lactic acid is produced by bacterial fermentation [4, 6].

The LAB isolation from fermented fish products, bekasam, which comes from a local processor in South Sumatra and Indramayu (West Java) and obtained 62 isolates of LAB. These sixty-two lactic acid bacteria have potential as an antimicrobial against some food pathogenic bacteria [7]. However, information about organic acid-producing LAB from bekasams which have antimicrobial activity in food is still lacking. Therefore, this study aimed to determined organic acids production and their antibacterial activity during LAB growth against of L. monocytogenes, S. typhimurium, E. coli, B. cereus dan S. aureus, to determine the organic acid contents and to identify the LAB.

2. Materials and Method
The study was done in the coastal area of South Sumatera Province, facing to Bangka Strait (Figure 1). Access to reach this area is only through waterway along the Musi River and coastal water area. Shoreline type is dominated by the muddy beach and inhabited by mangrove vegetation. This shallow water area is affected by high-organic sediment of the Musi River. The local community utilizes the area as their fishing ground with major fishing gears such as Kelong (a combination of Guiding Barrier and Fixed lift net) and Sero (Guiding Barrier). Four of lactic acid bacteria from bekasam (the BP(3), the BP(20), the BI(3) and the SK (5) strains) were grown in MRSB medium, incubated at 37 °C for 48 hours under microaerophilic conditions. Every 4 hours incubation, titratable acid total (TAT) was determined by titrating the fermented samples with NaOH 1 N (the Mohr methods), pH was determined by direct measuring with a pH meter (eutech instruments), LAB growth was numerated by a bacterial viability count and antibacterial activity using the agar well diffusion assay as previously described by according to Diop et al. modified [8]. After incubated for 48 hours, the culture was centrifuged and cell-free supernatant was analyzed for organic acid content using the High Performance Liquid Chromatography method [9].

Antimicrobial activity test was carried out of the cell-free supernatant using the agar well diffusion method against of Escherichia coli, Salmonella typhimurium ATCC 14 028, Staphilococcus aureus and Listeria monocytogenes. Twenty microliter of test bacterial which had cell density of 108 CFU/ml were suspended into 20 ml of MHA medium, then poured into a sterile petri dish. After MHA medium became solid, wells with a diameter of 5 mm was made. Seventy milliliters of cell-free supernatant were inserted into the well, then incubated for 24 hours at 37 °C. Clear zone which formed were then measured its diameter. For each of LAB isolates, isolate was performed in two duplicate.

Identification of LAB using molecular method based on 16S rDNA sequences. Genome extraction was carried out using a kit from Qiagen namely QIAamp DNA mini Kits catalog no 51304. The extraction procedure was carried out according to the company's protocol. Amplification of 16S rRNA
gene using Polymerase Chain Reaction (PCR) with universal primers for prokaryotes according to Marchesi et al. [10] namely 63F (5’CAG GCC TAA CAC ATG CAA GTC3’) and 1387R (5’GGG CGG GTG GTA CAA GGC3’) and DNA polymerase GoTaq master Mix (Promega). PCR conditions were as follows: initial denaturation was carried out at 95 °C for 5 minutes, followed by 30 cycles at 94°C for 30 seconds, 50 °C for 1 minute, 72°C for 2 minutes and then the final elongation at 72 °C for 5 minutes and 20 °C for 10 minutes. PCR products were confirmed by electrophoresis using 1% agarose gel and 1 x TAE buffer. PCR results were sequenced using ABI Prism 3100-Avant Genetic Analyzer (Applied Biosystems, USA). Analysis of DNA sequences was carried out using the BioEdit program, and the The National Center for Biotechnology Information (NCBI) Gen Bank (http://www.ncbi.nlm.nih.gov) using the Basic Local Alignment Search Tool for nucleotides (BLASTN) program. Furthermore, a phylogenetic tree was created using the Molecular Evolutionary Genetics Analysis (MEGA) version.

3. Results

3.1. Change of cell viability count and pH during growth of LAB
The LAB growth of the all four strains had the same pattern, namely the exponential phase occurred at 0-16 hours of incubation, while stationary phase occurred at 16-48 hours of incubation. Along with the increase in the growth of LAB, there was change in pH, which the pH decreased then at stationary phase tends to be stable. pH decreased to 3,4 in the SK (5) strain and 3,7 in the BI (3), the BP (3) and the BP (20) strains during 16 hours of incubation (Figure 1).

Figure 1. Relationship between changes in pH (♦) and LAB cell viability (log CFU/mL) (■) during 48 hours of incubation in the BI (3) (A), the BP (3) (B), the BP (20) (C), and the SK (5) (D) strains.

3.1.1. Production of organic acids during growth of LAB and its content
Production of organic acids for 48 hours of incubation in the four of LAB strains also had the same pattern such as changes in pH and LAB growth. It increased rapidly for 4 to 16 or 20 hours of incubation, after that TAT increased slowly to the end of incubation. The highest TAT concentration of 20-22 g / L in all four strains (Figure 2). The All four strains produced lactic acid, acetic acid and oxalate acid, in addition in the BP (20) and the SK (5) strains also produced formic acid, fumaric acid
and ascorbic acid. In case of the BP (3) and the SK (5) strain, production of lactic acid concentration produced was greater than that of acetic acid meanwhile the BI (3) and the BP (20) strains produced the acetic acid greater than the lactic acid (Table 1).

Table 1. The organic acids content of the BI (3), the BP (3), the BP (20), and the SK (5) strains.

| No | Organic acids   | Concentration (ppm) |
|----|-----------------|---------------------|
|    |                 | BI(3) | BP(3) | BP(20) | SK(5) |
| 1  | Formic acid     | nd    | 180,90| nd     | 188,09|
| 2  | Fumaric acid    | nd    | 139,50| nd     | 141,15|
| 3  | Acetic acid     | 739,11| 230,54| 628,48 | 249,65|
| 4  | Propionic acid  | nd    | nd    | nd     | Nd    |
| 5  | Tartaric acid   | nd    | nd    | nd     | Nd    |
| 6  | Citric acid     | nd    | nd    | nd     | Nd    |
| 7  | Oxalic acid     | 200,94| 19,50 | 53,07  | 230,88|
| 8  | Lactic acid     | 119,71| 747,47| 115,50 | 778,26|
| 9  | Ascorbic acid   | nd    | 8,01  | nd     | 13,74 |

nd: not detected, The detection limit of the instrument is 0.01 ppm.

3.2. **Antibacterial activity produced during the growth of LAB strains**

Antibacterial activity against all of the test bacteria produced by the BI(3) and the BP(20) strains were started at 8 and 12 hours of incubation, respectively, while the BP(20) and the SK(5) strains were different, ie at 4-12 hours of incubation which depends on species of the test bacteria (Figure 3). Antibacterial activity showed that the inhibition zone increased during the LAB growth, and the maximum inhibition zone occurred at the stationary phase with diameter of 8-15 mm. The highest antibacterial activity produced by the SK(5) strain against *B. cereus* of 15 mm (Figure 3).
3.3. Identification of LAB

The results of PCR amplification on the electrophoresis gel showed that the DNA bands of LAB isolates were between the bands 1000-1500 base pairs. The results of DNA sequencing of LAB isolates showed that BI (3), BP (3) and BP (20) strains had similarity of 98%, 97%, 98% with *Pediococcus pentosaceus* IE-3 (access code of CAHU01000036.1) respectively, while SK(5) strain was similar of 93% with *Lactobacillus plantarum* subsp. *plantarum* NC 8. (access code of AGR101000003.1). Dendogram of phylogenetic tree of LAB isolates can be seen in Figure 4.

4. Discussion

The result showed that the exponential phase of bacteria growth occurred at 16-20 hours incubation. Cohen [11] states that essential nutrient during the exponential phase was decrease, it is because of the medium was too acidic or alkaline. This condition will cause the growth rate of bacteria to decrease because the accumulation of toxic substances will able to inhibit cell division (the cell viability count remains constant).

Changes in pH occurred during the growth of the four LAB strains, pH decreased to 16 hours of incubation and after that the pH tended to be stable until the end of incubation (48 hours) (Figure 1). When the cells were in phase of exponential growth, the cells count increased rapidly so that the metabolic activity in the cells becomes higher. This cells metabolic activity produced organic acids, one of which was lactic acid. The percentage of lactic acid increases in the medium during 48 hours of incubation becomes acidic in the all four LAB strains of 3.29 to 3.56. The lowest pH reduction by the SK (5) strain, namely pH of 3.29 with a maximum lactic acid concentration of 21.60 g/L.
Figure 4. Dendogram of phylogenetic tree of LAB with bootstrap and aligned with Genbank.

The organic acid production of the LAB strains can be tracked unambiguously in the change of pH and titratable acidity. The pH decrease and the titratable acidity increase were greater in case of the better acid producer strains in given media. These changes of acidity just slightly correlated with the amount of produced total acids, but more correlated with the lactic acid production [12].

Production of organic acids (lactic acid) in all LAB strains increased during LAB growth. The dominant metabolites produced by all LAB strains were organic acids, such as lactic acid, acetic acid, and oxalic acid. The highest of TAT was 21.60 g / L produced by SK (5) strain for 44 hours of incubation. Similar results were also reported by Oalaoye and Onilude [13] that the product of lactic acid increased with the incubation time for all strains. The highest of lactic acid production were 23.37 and 28.02 (g / 107 CFU) produced by P. pentosaceus INT02 and P. pentosaceus INT01 for 42 hours of incubation, respectively.

The results showed that the greater lactic acid production (747.47 – 778.26 ppm) was in the BP(3) and SK(5) strains than in the BI(3) and the BP(20) strains, although using the same medium i.e the complex MRS broth medium. Zalan et al. stated that in this medium, the sole carbohydrate source is the glucose, which can be fermented by the LAB strains through two major pathways: the glycolysis (Embden–Meyerhof pathwy) is used by the homofermentative LAB, 1 glucose + 2 Pi → 2 lactate + 2 ATP + 1 H2O and the 6-phosphogluconate/phosphoketolase (6-PG/PK) pathway, 1 glucose + Pi +ADP → 1 lactate + 1 acetate +ATP+ 1CO2; is used by heterofermentative LAB. On the glucose substrate, the lactic acid is the main end product in both metabolic pathways [12].

The identification results showed that the SK(5) strain was Lactobacillus plantarum, while three LAB strains were Pediococcus pentosaceus. The four LAB strains were homolactic fermentation, using the glycolysis pathway (Embden-Meyerhof-Parnas pathway) in fermenting glucose to produce lactic acid. Glycolysis is characterized by the formation of fructose-1,6 diphosphate (FDP), which is
slipted by a FDP aldolase into dihydroxyacetonephosphate (DHAP) and glyceraldehyde-3-phosphate (GAP). GAP (and DHAP through GAP) are then converted to pyruvic in a metabolic sequence including substrate-level phosphorylation at two sites. Under normal conditions, i.e., excess sugar and limited access to oxygen, pyruvic is reduced to lactic acid by a NADH-dependent lactate dehydrogenase (nLDH), thereby reoxidizing the NADH formed during the earlier glycolytic steps. A redox balance is thus obtained, lactic acid is virtually the only end product [14].

The organic acid content produced by the four LAB isolates showed that lactic acid was the highest of organic acid produced by BP (3) and SK (5) strains with concentrations of 747.47 ppm and 778.26 ppm respectively. While the highest of organic acid produced by BP (20) and BI (3) strains were acetic acid with concentrations of 739.11 ppm and 628.48 ppm respectively (Table 1). The different of end product pattern produced by LAB showed that LAB adapted to various conditions and change their metabolism accordingly. LAB as a group exhibit an enormous capacity to degrade different carbohydrates and related compounds. Generally, the predominant end product was, of course, lactic acid (>50% of sugar carbon) [14].

The antibacterial activity produced by the four LAB strains increased until the 16-20 hours of incubation. This showed that the antibacterial activity was closely related to organic acid compounds produced during LAB growth. The maximum antibacterial activity produced in the stationary phase, which has accumulated metabolites produced by bacteria (Figure 3). Therefore, the antibacterial compounds produced by the four LAB isolates were organic acids, especially lactic acid (Table 1). The antibacterial activity produced by the four LAB isolates against of pathogenic bacteria was different (Figure 3). It was caused by antimicrobial compounds produced by each isolate were also different. The highest inhibition zone was produced by SK(5) strain against of B. cereus at 36 hours of incubation. This strain also showed the highest produced of organic acid compared to other strains. Therefore, the antibacterial compounds produced by the four LAB isolates were organic acids, especially lactic acid (Table 1). Singh (2018) state that the major antimicrobial compounds produced by LAB were bacteriocin, organic acids and hydrogen peroxide [1].

Lactic acid had shown antibacterial activity against spore-forming bacteria, but had little effect on fungi. Acetic acid and propionic acid generally inhibit the growth of other bacteria more effectively than lactic acid. Because higher pKa values can cause the possibility to diffuse through other cell membranes at higher pH. Both have been used extensively in the food industry as antibacterial additives [5,6]. The lactic acid is stronger acid (pKa = 3.86) than the acetic acid (pKa = 4.73) [12].

Lactic acid bacteria are generally defined as a group of lactic acid-producing, low% G + C, no spores, Gram-positive rods and cocci, fermentative, catalase-negative, facultative anaerobic, nonmotile and tolerant of acid [15]. Characteristic of the four LAB strains were grown on MRSA media supplemented with 0.1% CaCO3 and blue bromotimol (as an indicator of acid) showed a yellow colony and surrounded by a clear zone. This is caused by the presence of blue bromotimol indicator which changes the colour of blue to yellow with a change in pH and CaCO3 which dissolves due to acid to form a clear zone around the colony. The four LAB strains were Gram-positive, coccus and rod-shaped, non-endosporic, non-motile, catalase negative, and homofermentative (Data not shown).

Lactic acid bacteria are the dominant bacteria in some fermented products such as the bekasam, namely the fermented product from Thailand, Plaa-som, which was the dominant LAB in this product was Lactobacillus pp. (79%) and Pediococcus spp. (18%) [16]. However, Paludan-Muller et al. [17] found that the dominant LAB species isolated from plaa-som was identified as P. pentosaceus (42% of isolated strains).

5. Conclusions
Cell counts of lactic acid bacteria and TAT increased but pH decreased during growth in all four LAB strains. Antibacterial activity against of the test bacteria were produced by all four LAB strains during growth and reach the highest activity at stationary phase. The highest of TAT was produced by SK (5) strain. The highest of organic acid content produced by BP (3) and SK (5) strains were lactic acid, while in the BP (20) and BI (3) strains were acetic acid. The BI (3), BP (3) and BP (20) strains were
Pediococcus pentosaceus with 98%, 97% and 98% similarities, respectively, while the SK (5) strain showed 94% similarity with Lactobacillus plantarum subsp. Plantarum.

Acknowledgement
The study team would like to express our sincere appreciation to the local community in the Coastal area of Banyuasin District for their invaluable collaboration support during the field survey. Special gratitude we give to all whose contribution in supporting the implementation of this research.

References
[1] Singh F V 2018 Recent approaches in food bio-preservation - a review Veterinary Journal 8(1) 104-111
[2] Nath S, Chowdhury S, Sarkara S and Dora K C 2013 Lactic acid bacteria – A potential biopreservative in sea food industry International Journal of Advanced Research 1(6) 471-475
[3] Khan I, Qayyum S, Maqbool F, Rehman M, Hayat A and Farooqui M S 2017 Microbial organic acids production, biosynthetic mechanism and applications -Mini review Indian Journal of Geo Marine Sciences 46(11) 2165-2174
[4] Singh R, Mittal A, Kumar M and Mehta P K 2017 Organic acids: An overview on microbial production International Journal of Advanced Biotechnology and Research 8(1) 104-111
[5] Ray R C and Joshi V K 2015 Fermented Foods: Past, Present and Future In Microorganism and Fermentation of Traditional Food Ray RC and Monted D (Eds.) (Boca Raton: CRC Press. p 2)
[6] Theron M M and Lues J F R 2011 Organic Acids and Food Preservation (New York: CRC Press. p 273)
[7] Desniar, Rusmana I, Suwanto A and Mubarak N R 2013 Characterization of lactic acid bacteria isolated from an Indonesian fermented fish (bekasam) and their antimicrobial activity against pathogenic bacteria Emirates Journal of Food and Agriculture 25(6) 489-494
[8] Diop M B et al. 2007 Bacteriocin producers from traditional food products Biotechnol Agron Soc Environ 11 275-281
[9] Adnan M 1997 Teknik Kromatografi untuk Analisis Bahan Makanan (Yogyakarta: Penerbit ANDI) hlm 109-120
[10] Marchesi J R et al. 1998 Design and evaluation of useful bacterium-specific PCR primers that amplify genes coding for Bacterial 16S rRNA Appl Environ Microbiol 64 795-799
[11] Cohen G N 2011 Microbial Biochemistry Second edition (London: Springer Science) p 7-10.
[12] Zalan Z, Hudacek J, Stetina J, Chumchalova J and Halasz A 2010 Production of organic acids by Lactobacillus strains in three different media Eur Food Res Technol 230 395-404
[13] Olaye O A and Onilude A A 2011 Quantitative estimation of antimicrobials produced by lactic acid bacteria isolated from Nigerian beef Int Food Research J 18 1155-1161
[14] Axelsson L 2004 Lactic Acid Bacteria: Classification and Physiology In Salminen S, Wright SV, Ouwehand A. (Eds.) Lactic Acid Bacteria. Microbiological and Functional Aspects Third edition, Revised and Expanded (New York: Marcel Dekker, Inc.) hlm 19-68
[15] Hutkins R W 2006 Microbiology and Technology of Fermented Foods (Lowa: IFT Press. Blackwell Publishing Ltd.) p 3-49
[16] Kopermsub P, Vichitphan S and Yunchalard S 2006 Lactic acid bacteria isolated from Plaasom, a Thai fermented fish product Thai J Biotechnol 7 32-39
[17] Paludan-Muller C, Madsen M, Sophanodora P, Gram L and Møller PL 2002 Fermentation and microflora of plaasom, a Thai fermented fish product prepared with different salt concentrations. Int J Food Microbiol 73 61–70