BIODIVERSITAS
Volume 20, Number 12, December 2019
Pages: 3532-3538

ISSN: 1412-033X
E-ISSN: 2085-4722
DOI: 10.13057/biodiv/d201210

Antimicrobial potential of *Pediococcus acidilactici* from Bekasam, fermentation of sepat rawa fish (*Tricopodus trichopterus)* from Banyuasin, South Sumatra, Indonesia

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Manuscript received: 7 October 2019. Revision accepted: 14 November 2019.

Abstract. *Melia S, Purwati E, Kurnia Y. F, Pratama D. R. 2019. Antimicrobial potential of* Pediococcus acidilactici *from Bekasam, fermentation of sepat rawa fish (*Tricopodus trichopterus*) from Banyuasin, South Sumatra, Indonesia. Biodiversitas 20: 3532-3538.* This study aimed to determine the antimicrobial potential of lactic acid bacteria isolated from bekasam. Bekasam is a result of sepat rawa fermentation from Banyuasin District, South Sumatra, Indonesia. The results showed that the morphological and biochemical properties of lactic acid bacteria were Gram-positive and cocci, negative catalase and included in homofermentative groups. The biggest antimicrobial activity was shown by bekasam isolate to *Escherichia coli* O157: H7 (21.26 mm), followed by *Staphylococcus aureus* ATCC25923 (18.23 mm) and *Listeria monocytogenes* CFSAN004330 (5.10 mm), while diameter barriers for crude bacteriocin supernatant isolates lactic acid bacteria to *Escherichia coli* O157: H7, *Staphylococcus aureus* ATCC25923 were 14.99 mm, 17.69 mm, and *Listeria monocytogenes* CFSAN004330 had no antimicrobial activity at neutral pH. The results of molecular identification with 16S rRNA showed that lactic acid bacteria isolated from bekasam isolate have similarity with *Pediococcus acidilactici* strain PB22 that has antimicrobial potential against pathogenic bacteria and potential as bio preservatives.

Keywords: Antimicrobial, bekasam, fermented fish, lactic acid bacteria, sepat rawa

INTRODUCTION

Traditional fermented natural food is very diverse in Indonesia because the territory of Indonesia is very wide and has distinctive food characteristics for each region one of them is curd. Curd is a natural fermentation of buffalo milk from West Sumatra that is beneficial for health (Surono 2003, 2009) and contains several types of lactic acid bacteria (Venema and Surono 2019). *Lactococcus lactis* ssp. *lactis, L. plantarum* ssp. *plantarum, L. lactis* ssp. *cremoris, Pediococcus pentosaceus,* and *Lactobacillus pentosus* are some types of bacteria that are naturally found in curd (Wirawati et al. (2019). Tempoyak, a natural fermentation product from Durian, is also a natural fermentation product (Juliyarsi et al. (2018) and tempoyak also contains lactic acid bacteria that have the potential as probiotics (Hartini et al. 2019 and Ahmad et al. 2018). In addition, there are also naturally occurring fish fermented products called Budu originating from West Sumatra. Lactic acid bacteria from Budu that have the potential as antimicrobial *Bacillus cereus* strain HVR22 (Yusra et al. (2013). All-natural fermentation product contains lactic acid bacteria that are very beneficial for health.

Bekasam is a traditional food originating from several regions in Indonesia such as Java, South Sumatra, and South Kalimantan. Bekasam is the result of spontaneous fermentation of fish. According to Desniar et al. (2013), Bekasam is used as a processed fish product by fermentation that tastes sour. Fish that can be used as exam is the type of freshwater fish. The raw material in the form of cork fish, beam, siam and swamp spikes with the addition of salt about 15-20%, and added 15% sangria rice, then fermented for about one week to produce a distinctive aroma and taste.

There are several previous studies about the content of lactic acid bacteria in bekasam. Wikandari et al (2012), found lactic acid bacteria that have proteolytic activity namely *Lactobacillus plantarum* B765, *L. plantarum* T2565, *L. plantarum* N2352, *L. plantarum* B1465, *L. pentosus* B2555, and *Pediococcus pentosaceus* B1666. Desniar et al. (2013), in their study, revealed the presence of antimicrobial activity of lactic acid bacteria isolates to *Staphylococcus aureus*, which was caused by the ability of organic acids as antibacterial compounds. Then, Afriani et al. (2015) isolated lactic acid bacteria of bekasam from Jambi, which also had proteolytic activity, namely *Lactobacillus pentosus* BS15, *L. plantarum* 1 BS22 and *L. plantarum* 1 BL12. Melia, et al. (2018), tested the antibacterial activity of lactic acid bacteria Bekasan against *Staphylococcus aureus* ATCC 25923, *Escherichia coli* ATCC 25922 and *Salmonella* sp. However, the previous researches do not provide the same type of fish that might be resulted in differences in microbial profiles compared to this study. This study will evaluate the potential of lactic acid bacteria from the fermented sepat rawa fish (*Tricopodus trichopterus* Pallas, 1770) that is a typical type of fish used in the Banyuasin region, which has antimicrobial

activity and is a potential source bacteriocin, which can later be used as probiotic and biopreservative.

MATERIALS AND METHODS

Bekasam Processing

The process of making bekasam is very simple. The ingredients consist of fish, rice, and salt. The fish was cleaned, then added salt and rice sufficiently, put in a bottle and closed tightly. The bottle was stored at room temperature for 3 days (Figure 1).

Sampling

The material of this study was 4 bekasam samples originating from sepat rawa fermented fish obtained from four producers in Banyuasin, South Sumatra, Indonesia (1,3°-4°S, 103°-105° E.) (Figure 2), namely Pulau Harapan Village (Producer 1), Mainan Village (Producer 2), Sei Rengit Village (Producer 3) and Santan Sari Village (Producer 4). This location is a swampy area that produces lots of Sepat rawa fish.

Isolation and Identification of Lactic Acid Bacteria

The isolate of lactic acid bacteria from bekasam was cultured in broth *De Man, Rogosa and Sharpe (MRS), broth* media (Merck, Germany) and planted in MRS agar media (Merck, Germany) that was incubated at 37ºC in an anaerobic jar for 48 hours. Furthermore, morphological properties (shape and color) were observed, and biochemical properties (Gram staining, catalase test and fermentation type) Phikunthong and Yunchalard (2010).

Figure 1. Bekasam processing: A. Clean sepat fish scales, B. Give salt and rice, C. Store in jar bottles, D. Bekasam is fermented for 3 days at room temperature

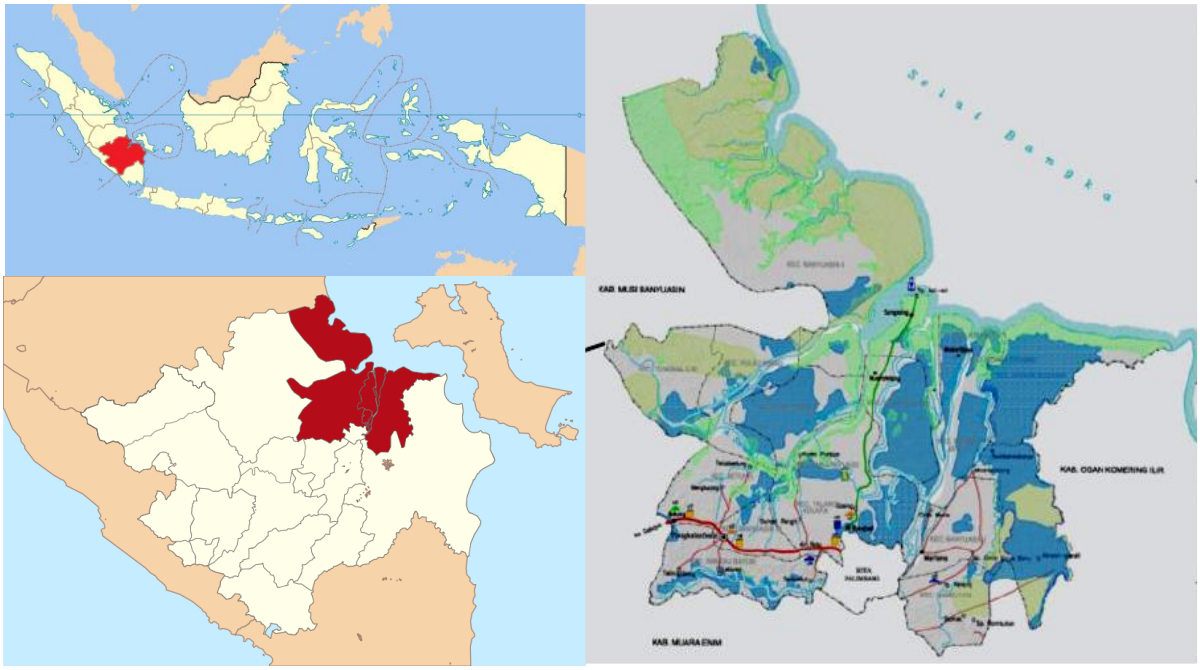


Figure 2. Study area in Banyuasin, South Sumatra, Indonesia (1,3°-4°S, 103°-105° E)

Antimicrobial activity test

Modification of the Yang et al. (2012) method was used for antimicrobial activity tests against pathogens. The method used was well diffusion assay. In short, cell-free supernatants were obtained from lactic acid bacteria grown in MRS Broth, for 24 hours at 37 °C, anaerobic conditions and centrifuged at 10,000 rpm, 5 minutes at 4°C. A 50 µL supernatant was inserted into a well (6 mm) that was perforated with a cork borer. Previously Nutrient Agar (Merck, Germany) has been grown by pathogenic bacteria. Pathogenic bacteria are grown aerobically at 37ºC for 24 hours. Then 0.2% of pathogenic bacterial culture was added into Nutrient Agar. As a control, it was compared to antibiotics (penicillin 10 µg, kanamycin 30µg, ampicillin 10µg). The clear zone formed can be read after 24 hours.

Antimicrobial test of crude bacteriocin supernatant

One ml of culture was incubated for 24 hours in 9 ml MRS broth for 24 hours at 37 °C. Then centrifuged at 14,000 rpm for 5 minutes. The supernatant was filtered with a 0.22 µL membrane filter. Cell-free supernatant was regulated up to pH 6.5 with 1 N NaOH to eliminate the effect of barriers because of the presence of organic acids (Yang et al. 2012). Pathogenic bacteria were grown aerobically at 37ºC for 24 hours. Then the pathogenic bacterial culture was 0.2% into 20 ml of Muller Hinton Agar (MHA) at 50 ºC. After agar became solid, well was with a size of 6 mm using the cork borer. Then the supernatant was taken as much as 50 µL and inserted into each well and allowed to stand for 15-20 minutes, then incubated for 24 hours at 37ºC in aerobic conditions. The inhibitory zone was measured by using the caliper. If the well is found, the inhibition zone can be said to be BAL isolate that contains bacteriocin compounds.

DNA genomes isolation of Lactic Acid Bacteria and 16S rRNA

Lactic acid bacteria isolates were cultured in MRS broth at 37°C for 24 h. Isolation of genomic DNA was carried out using Promega Kit (USA). Single colony lactic acid bacterial isolates from MRS Broth were piped as much as 1000 µL and included in the new Eppendorf. Centrifuged as 14000 rpm for 2 minutes. Then the supernatant is removed and the pellet is taken. Added with 480 µL 50 mM EDTA. Then, 120 µL of Lysozyme was added. Next, Incubation in 37ºC water bath for 60 minutes. Centrifuge for 2 minutes 14000 rpm, then remove the supernatant and pellet is taken. Added with 600 µL nuclei lysis solution. Incubated 80ºC for 5 minutes, then let it stand at room temperature. Added 3µL of RNase Solution, incubated in water bath 37ºC for 60 minutes. Added with 200 µL of the protein precipitation solution then vortex. 600 µL of isopropanol was added. Centrifuged for 2 minutes 14000 rpm, then pellets are taken and the supernatant is removed. Added 600 µL of ethanol 70% and then homogenized. Centrifuged for 2 minutes 14000 rpm, then pellets were taken and the supernatant was removed. Pellet DNA rehydration by adding 10-100 µL of Rehydration solution for 30 minutes at 65ºC. Primer R (16S-1492R, Tm 47°C, 5'-GTT TAC CTT GTT ACT ACT-3') and F (16S-27F, Tm 54.3°C, 5'-AGA GTT TGA TCC TGG CTC AG-3'), prepared (concentration of 10 pM). Take 90µL dH2O + 10µL (Primary R and F). (Primary R and F in TE buffer (concentration 100 µM). Cocktail PCR in1 Eppendorf (Master Mix 12.5 µL, Primary F 1 µL, Primary R 1 µL, Template DNA 1 µL, ddH2O 9.5 µL), with PCR denaturation 95°C 45 seconds, annex 56°C 45 seconds, Extention 72°C 1 minutes 40 seconds, final extention72°C 10 minutes. Electrophoresis of 10 µL samples into the well agar, inserted 4 µL of the DNA ladder. Set to 100 V for 45 minutes. The gel placed in a container plus TBE until submerged. The gel, then seen under the UV lamp. The 16S rRNA gene sequences of the isolate were submitted to the NCBI for a BLAST search. The MEGA version 6.0 (http://www.megasoftware.net) was used to create phylogenetic trees using the neighbor-joining (NJ) method.

RESULTS AND DISCUSSION

Morphological and biochemical characteristics of lactic acid bacteria isolate

Fifty-six isolates of lactic acid bacteria were isolated from bekasam, morphologically round (cocci), rod-shaped, and cream-colored. The testing of biochemical properties showed that the results of Gram-positive and negative catalog as well as isolated of lactic acid bacteria were homofermentative as indicated by the absence of gas bubbles in the Durham tube. Desniar et al. (2013), also stated that in general, the LAB isolates from bekasam were homofermentative.

Antimicrobial activity of Lactic Acid Bacteria isolate

Of the fifty-six isolates of lactic acid bacteria, there were 4 isolates that had antimicrobial activity, but only bekasam isolates had antimicrobial activity against the three tested pathogenic bacteria, *Escherichia coli* O157: H7, *Staphylococcus aureus* ATCC25923 and *Listeria monocytogenes* CFSAN004330. *Antimicrobial activity* of bekasam isolates from bekasam on pathogenic bacteria, *Escherichia coli* O157: H7, *Staphylococcus aureus* ATCC25923 and *Listeria monocytogenes* CFSAN004330 can be seen in Table 1. Antibiotics used positive controls (Penicillin 10µg, Ampicillin 10µg and Kanamycin 30µg).

In Table 1, the largest antimicrobial activity was shown by bekasam isolate against *E. coli* O157: H7, with a clear zone diameter of 21.26 mm followed by *S. aureus* ATCC 25923 18.23 mm and *L. monocytogenes* CFSAN004330 5.10 mm. LAB isolate, had greater antimicrobial activity against *E. coli* O157: H7 than the three antibiotics used, namely penicillin 10 µg, kanamycin 30 µg, ampicillin 10 µg.

Table 1. Antimicrobial activity bekasam isolate and antibiotic test

Inhibitory source	Clear zone (mm)		
	Escherichia coli **O157: H7**	***Staphylococcus aureus*** **ATCC 25923**	***Listeria monocytogenes*** **CFSAN004330**
Isolate LAB	21.26 ± 0.03	18.23 ± 0.01	5.10 ± 0.01
Penicillin10µg	2.70 ± 0.03	-	-
Ampicillin10µg	14.19 ± 0.05	21.26 ± 0.02	-
Kanamycin 30µg	16.21 ± 0.09	13.18 ±0.05	10.15 ±0.08

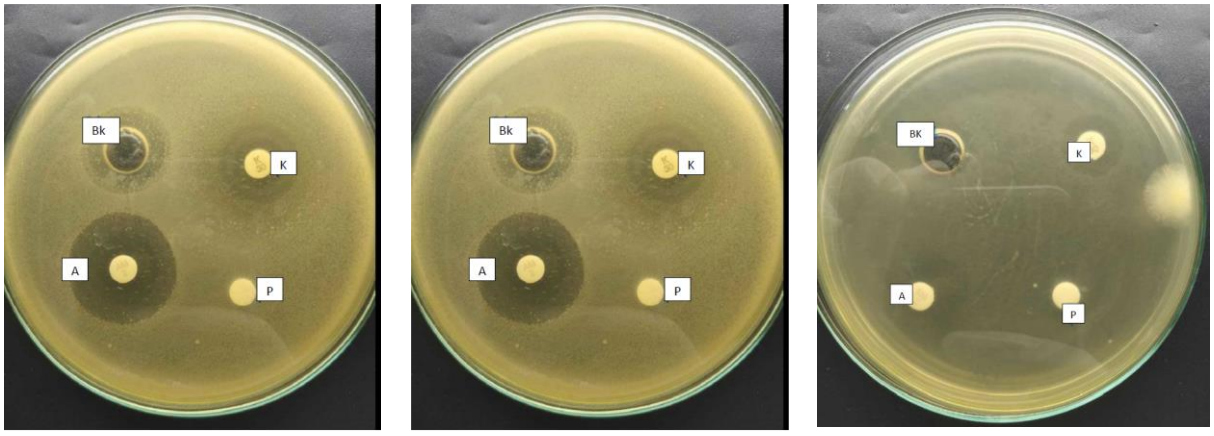
Note: The value is expressed as the mean ± standard deviation; n=3

However, as a whole in Figure 3, it can be seen that bekasam isolate from the stain has antimicrobial activity against all pathogenic bacteria, compared with penicillin which does not inhibit growth of *S. aureus* ATCC 25923 and *L. monocytogenes* CFSAN004330 and kanamycin had no antimicrobial activity to *L. monocytogenes* CFSAN004330. Desniar (2012) states that the antimicrobial test against the BALs of tilapia extracts has the ability to inhibit five types of pathogenic bacteria: *E. coli*, *S. typimurium* ATCC 14028, *Bacillus aureus, S. aureus,* and *L. monocytogenes*. The results showed that inhibited zones on pathogenic bacteria had high antimicrobial activity. Pan et al (2009) state that the diameter of the inhibited zone against 0-3 mm pathogenic bacteria showed low antimicrobial activity that is > 3-6 mm medium antimicrobial activity and > 6 mm had high antimicrobial activity. Liasi et al. (2009) stated the antagonistic effect of antibacterial compounds on gram-positive and gram-negative pathogenic microbial bacteria, such as *E. coli*, *L. monocytogenes*, *Salmonella enterica*, *Staphylococcus aureus*, and *Bacillus cereus*.

The same result is also shown by Saithong et al. (2010), using *L. reuteri* IFRDP P17 in Plaa-som, a typical Thai fish fermentation product, capable of suppressing growth, but in contrast with Desniar et al. (2013), which states that isolate LAB from exteriors from Indralaya, Ogan Komiring Ilir (South Sumatra) and Indramayu (West Java), the largest activity of lactic acid bacteria antimicrobials was against *S. aureus*. Lactic acid bacteria were the dominant bacteria found in fermented fish products (Olympia et al. 1992; Ostergaard et al. 1998). Melia, et al. (2017) states that adding lactic acid bacteria is able to inhibit *L. monocytogenes*. It can also inhibit *S. aureus* ATCC 25923 (Melia, et al. 2018). The main role of lactic acid bacteria is to ferment carbohydrates that produce organic acids, which can lead to a decrease in pH. The low pH and presence of organic acids, the main is lactic acid, is a major factor in the process of preservation in fermented fish products. Generally, pH between 4.5-5.0 can inhibit pathogenic bacteria and decomposers (Owen and Mendoza, 1985). Organic acid produced by Lactic Acid Bacteria has antibacterial activity (Theron and Ludes 2011). Deatraksa et al. (2018) have isolated and identified *weissella* strains from Thai fermented fish (Plaa Som Fug) which produces antibacterial and folate compounds.

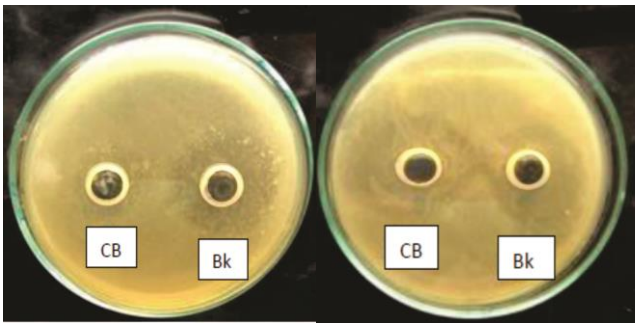
Antimicrobial activity of crude bacteriocin supernatant

The measurement of antimicrobial activity of isolates Bekasam crude bacteriocin supernatant was obtained after neutralizing pH in the supernatant of lactic acid bacteria, so that the antimicrobial activity of organic acid was not present. According to Palludan-Muller et al. (2002), components of organic acids, especially lactic acid is the main components of the antimicrobial compounds of lactic acid bacteria. The results of the study can be seen in Table 2 that showed antimicrobial activity after pH of the lactic acid bacterial supernatant was neutralized to the *E. coli* O157: H7 was 14.99 mm and *S. aureus* ATCC25923 that was 17.69 mm (Figure 4), but defention activities were not shown to *L. monocytogenes* CFSAN004330.



A **B** **C**

Figure 3. Antimicrobial activity of bekasam isolates to *E. coli* O157: H7 (A), *S. aureus* ATCC 25923 (B) and *L. monocytogenes* CFSAN004330 (C). (Note: Bk = Isolate LAB Bekasam, A = Ampicillin, K = Kanamycin, and P = Penicillin)



A **B**

Figure 4. Antimicrobial activity of crude bacteriocin after neutral pH (CB) and before neutral pH (Bk) against *E. coli* O157: H7 (A), against *S. aureus* ATCC 25923 (B)

Table 2. Antimicrobial activity of bekasam isolate crude bacteriocin supernatant

Pathogenic bacteria	Diameter clear zone (mm)
E. coli O157: H7	14.99 ± 0.03
S. aureus ATCC 25923	17.69 ± 0.01
L. monocytogenes CFSAN004330	-

Note: The value is expressed as the mean ± standard deviation; n=3

The result was higher than Melia, et al. (2018) study. In their study, the crude bacteriocin LAB isolates activity from bekasam was against *S. aureus* ATCC 25923 (13.1 mm) and *E. coli* O157: H7 (12.7 mm). Whereas in the Desniar et al. (2013), LAB isolates from bekasam did not have antimicrobial activity after supernatant pH was neutralized so that it was thought, antimicrobial activity originated from organic acids produced by lactic acid bacteria. Furthermore, Desniar et al. (2016) isolated *L. plantarum NS* (9) from Bekasam Tilapia Atin that produced antibacterial activity from organic acids. The highest antibacterial activity against E. *coli, B. cereus* and *L. monocytogenes* at the end of the exponential growth phase (12-15 hour incubation) while *S. aureus* and *S. typhimurium* ATCC 14028 on the 21st and 24th incubation hours.

Furthermore, Fall et al. (2018), revealed that the antimicrobial activity of supernatant cell-free culture from *Lactobacillus plantarum* and *L. brevis* isolated from fermented fish meat (guedj) in Senegal was able to inhibit *E. coli* and *L. monocytogenes*. Srionnual et al. (2007), found that Weissellicin 110, a class II bacteriocin produced by Weissellicin 110 isolated from Pla-som was able to inhibit gram-positive bacteria, but did not have antimicrobial activity against *Listeria monocytogenes.* Nurhikmayani et al. (2010), crude bacteriocin from lactic acid bacteria was isolated from Chao, against *S. aureus* FNCC0047 and *E. coli* FNCC0049.

According to Islam et al. (2012), there are several mechanisms to inhibit the destruction of target cells by bacteriocins. Basically inhibiting the formation of lipids II (cell wall precursors) can be done by following mechanism L a. Inhibition of cell wall biosynthesis, b. Stabilize the formation of membrane target pores. Added by Bahar, and Ren (2013), and Song and Zheng (2015)that when the peptide attaches the target cell membrane, the positive end of the peptide will bind the fatty acids in the phospholipid layer on the target bacterial membrane. This stage involves binding the peptide with a membrane-like a monomer, so that separation occurs that leads to the formation of pores, ultimately causing death in the cell.

Results of 16S rRNA gene amplification by PCR

In Figure 5, it can be seen that the amplification of the area of the 16S rRNA gene isolates lactic acid bacteria from bekasam. It can be seen by the appearance of PCR fragment of size 1542 bp using R Primer (16S-1492R, Tm 47 °C, 5'-GTT TAC CTT GTT ACT ACT-3´) and F (16S-27F, Tm 54.3 °C, 5'-AGA GTT TGA TGCC CTC AG-3´)(Doi et al. 2013).

Phylogenetic trees based on 16S rRNA gene sequence analysis can be seen in Figure 6. Sequencing results of bekasam isolates compared to Gene Bank data using the BLAST program on the NCBI website (http://www.ncbi.nlm.nih.gov) showed a similarity rate of 99% with PB22 strain *Pediococcus acidilactici*, so it can be concluded that the lactic acid bacteria isolate from bekasam isolate is *P. acidilactici* strain PB22. This isolate lactic acid bacteria is a new strain found in bekasam or other fermented fish.

Afriani et al. (2015), isolated lactic acid bacteria from Bekasam from Jambi, which also has proteolytic activity, namely *Lactobacillus pentosus* BS15, *Lactobacillus plantarum* 1 BS22 e *Lactobacillus plantarum* 1 BL12. Nurhikmayani et al. (2019), found *Lactobacillus plantarum* and *Pediococcus pentosaceus* from Chao fermented fish from South Sulawesi using 16S rRNA.

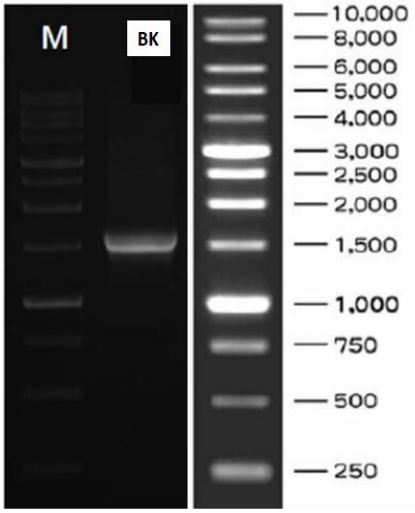


Figure 5. The PCR amplification of ribosomal RNA gene using l1492R and 27F. BK is isolated lactic acid bacteria bekasam. (M = 1 kB DNA Ladder)

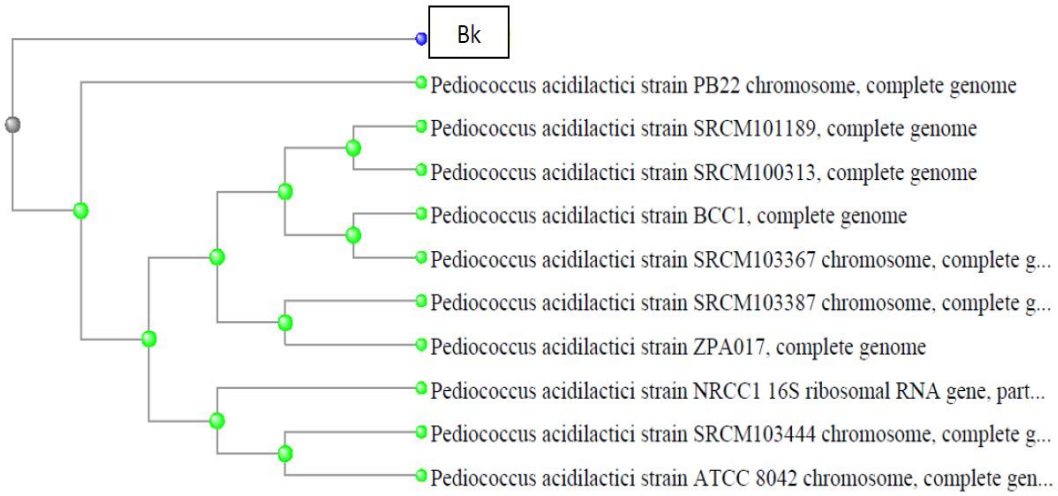


Figure 6. Phylogenetic isolate of lactic acid from bekasam (Bk) (Neighbor-Joining tree-1000x bootstrap)

In addition there are also several sources of lactic acid bacteria from Thai Plaa-som fermented fish products such as *Pediococcus pentosaceus, Lactobacillus alimentarius/ farciminis, Weisella confusa, L. plantarum* and *Lactococcus garviae* from Plaa-som, fermented fish products from local producers in Songkhla province, Southern Thailand (Paludan-Mu¨ller et al. 2002), *Lactococcus garvieae, Streptococcus bovis, Weissella cibaria, Pediococcus pentosaceus, Lactobacillus plantarum,* and *Lactobacillus fermentum* (Kopermsub and Yunchalard 2010), *Lb. plantarum* and *Pediococcus pentosaceous* (Nicomarat et al. 2018).

In summary, the results of molecular identification with 16S rRNA showed the potential lactic acid bacteria (bekasam isolate) as antimicrobial isolation from Bekasam from South Sumatra Banyuasin was similarity with *P. acidilactici* strain PB22. *P. acidilactici* strain PB22, is the new strain found in bekasam. Furthermore, *P. acidilactici* can later be used biopreservatives.

ACKNOWLEDGEMENTS

This research was supported by research cluster publications to professors (Project T/6/UN.16.17/PP-KP-KRP2GB/LPPM/2019) LPPM Universitas Andalas, Padang, Indonesia.

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