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ANTIBACTERIAL POTENCY OF CELL FREE SUPERNATANT PRODUCED BY LACTIC ACID BACTERIA ISOLATED FROM INDONESIAN FERMENTED FISH PRODUCTS AGAINST HISTAMINE-PRODUCING BACTERIA

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ABSTRACT

The toxicity caused by high histamine content produced by histamine-producing bacteria (HPB) during the fermentation of scombridae fish group based fermented fish products is still a problem that requires a solution. This study aims to explore the potential of secondary metabolites in the form of cell free supernatant (CFS) produced by lactic acid bacteria (LAB) as antibacterial agents against HPB. The LAB was isolated from the fermented fishery products named bekasan, cincalok and fish sauce using MRS-Agar and fermented on MRS-broth for 48 hours. CFS was collected by centrifugation at 15,000 x g for 15 minutes, followed by heating at 100^{o}C for 3 minutes and pH neutralization with 0.1N NaOH. Antimicrobial activity of CFS then tested on HFB namely *Morganella morganii* TK7, *Citrobacter freundii* CK1, and *Klebsiella* sp. CK13.2 (collection of the Laboratory of Fisheries Product Quality and Safety, Department of Fisheries UGM) using the macrodilution method. Isolation of LAB from all three products successfully isolated 34 isolates. The results of the antibacterial activity showed that 4 isolates namely GMCN 1.12, GMBK 2.6, GMBK 2.7, dan GMKI 2.1 were able to inhibit HFB growth more than 70%. The highest activity was shown by GMBK 2.7 which inhibits 98% against *Morganella morganii* TK7, 99% against *Citrobacter freundii* CK1, and 84% against *Klebsiella* sp. CK13.2. The antimicrobial activity was reduced after proteolytic enzymes were added suggesting that the bioactive compound came from peptide-based substances like bacteriocin.

Keywords: antibacterial; lactic acid bacteria; histamine-producing bacteria; cell free supernatant

INTRODUCTION

Histamine is known as an active toxin that poisons the body by means of intoxication. Food poisoning due to the formation of histamine in fish products is called scombroid fish poisoning due to histamine being mostly formed by fish from the Scombridae family. Histamine is classified as biogenic amine with amine chains derived from amino acids by enzymatic reactions which is formed due to the decarboxylation of the amino acid histidine into histamine by enzyme L-histidine-decarboxylase. The occurrence of histamine in fish and fish products is mainly produced by histamine-producing bacteria (HPB) including *Morganella*, *Klebsiella*, *Roultella*, *Serratia* and other Enterobacteriaceae (Ababouch *et al*., 1991). The toxicity of histamine mainly occurs due to an allergic reaction in the body with an inflammatory response to organs exposed to allergies such as itching, shortness of breath, and swollen glands (Wakinaka *et al*., 2019). The U.S. Food & Drug Administration (FDA) states that the histamine content of 50 mg / kg in fishery products is capable of causing poisoning effects.

In Indonesia, various kinds of fermented fishery products are made from Scombridae class fishes such as skipjack, tuna, and other fish. The utilization of this type of fish allows the formation of histamine which can cause intoxication (Heruwati *et al*., 2004). Histamine content in fermented fish product have been reported in various product such as fish sauce, fish paste and shrimp paste from Taiwan (Tsai *et al*., 2006), fish sauce (Mooraki and Sedaghati, 2018), Rihaakuru (a cooked fish paste from Maldive) (Naila *et al*., 2011), and Padaek (fish paste from Laos) (Marui *et al*., 2021).

Handling techniques to prevent the formation of histamine in fishery fermentation products can be done by maintaining the quality of fish used or by adding histamine degrading enzymes like Diamine oxidase (Naila *et al*., 2011) and / or adding Lactic acid bacteria (LAB) metabolites which have antibacterial activity against histamine-producing bacteria (Lim, 2016). The addition of histamine-degrading enzymes to fermented fishery products is easy to do, but it is expensive whereas adding LAB which can produce metabolite compounds with antibacterial potency is more applicable. Bacteriocin is known as one of BAL extracellular metabolites which is known as GRAS (generally recognized as safe) and can be applied in fishery fermentation products. Bacteriocins are able to cause other bacterial cells to undergo lysis so that there is a change in the permeability of the cytoplasmic membrane and deactivate the work of enzymes in the cell (Gautam and Sharma, 2009). Lactic acid bacteria that grow in the product fermentation process have a fairly high diversity. This is influenced by a favourable growing environment and the nature of lactic acid bacteria which are tolerant of salt and

acids. The formation of bacterial groups in the fermentation process causes competition between bacterial colonies. The competition includes the production of molecules that can inhibit the growth of other bacteria (Bayona and Comstock, 2018). One of the molecules often produced by lactic acid bacteria that can inhibit the growth of other microbes is bacteriocin (Ueda and Beppu, 2017).

The potency of bacteriocin from LAB against HPB has been reported. Lim (2016) reported that the type of BPH isolated from myeolchi-jeot products (a type of salted anchovies) can be inhibited by antibacterial products produced by LAB with evidence of decreased production of histamine. This inhibition occurred since the strains of lactic acid bacteria *Pediococcus acidilactici* MCL11, *Leuconostoc mesenteroides* MCL12, *Enterococcus faecium* MCL13, *Lactobacillus sakei* MCL14, and *Lactobacillus acidophilus* MCL15 were reported to produce antibacterial compounds. Other research reported that *Lactococcus lactis* produces bacteriocin (nisin) which can inhibit the growth of HPB such as *Lactobacillus buchneri* and *Lactobacillus brevis* (Josten and Nunez, 1996). However, the research on potency of metabolites produced by LAB isolated from Indonesian local fermented fish products against HPB is not yet explored. Therefore, this research was done to screen the LAB from Indonesian local fermented fish products namely bekasam, cincalok and fish sauce and study the antibacterial potency in form of cell free supernatant against HPB. This research is expected to find lactic acid bacteria that have the potential to be used as a starter in the fermentation process and function to reduce the histamine content in the fermented product.

RESEARCH METHODS

Materials

The traditional fermented products, bekasam, cincalok and fish sauce, were purchased online from South Sumatera, West Kalimantan and Centra Java respectively. The histamine-forming bacteria isolates used in this study namely *Morganella morganii* (TK7), *Klebsiella pneumoniae* (CK13.2), and *Citrobacter freundii* (CK1) were the cultures collection of Fisheries Product Quality a and Safety Laboratory, Department of Fisheries, Faculty of Agriculture Universitas Gadjah Mada.

The media used are de Man Rogosa and Sharpe (MRS) broth and agar (Sigma Aldrich), Trypticase soy broth and agar (Sigma Aldrich), 0.85% NaCl (Oxoid), 30% glycerol, CaCO3 (Merck) and commercial papain. This study also used supporting materials including 70% alcohol, 10% NaOH, distilled water, H_2O_2, KOH 3%, Gram staining kit (Sigma Aldrich), and kanamycin (Meiji).

Tools used are Petridis, micropipette, yellow tip, blue tip, micro tube, microscope (Olympus Model BX51TRF, Olympus Optical Co., Ltd., Tokyo, Japan), vortex (Barnstead M37610-33), refrigerator -30 ◦C (Sanyo SR-D180F), incubator (Isuzu SSJ-115), centrifuge (Kokusan H-26 F), hot plate stirrer (Nuova Stir Plate), autoclave (Hi-clave HVE-50 Hirayama), magnetic stirrer, and spectrophotometer UV-Vis (Genesys 10s UV-Vis).

All experiments were done at Laboratory of Fisheries Product Quality and Safety, Department of Fisheries, Faculty of Agriculture Universitas Gadjah Mada.

Methods

Isolation of Lactic Acid Bacteria (LAB) from Fermented Foods

Lactic acid bacteria isolation from bekasam and cincalok were conducted according to Abrams *et al.* (2011) while Udomsil *et al*. (2010) was used for lactic acid bacteria isolation from fish sauce. Sample preparation from solid products (bakasam and cincalok) was performed by dissolving 10 g of each product in 90 ml 0.85% NaCl solution, while sample from liquid source (fish sauce) was carried out by dissolving 1 ml of fish sauce in 9 ml 0.85% NaCl solution under aseptic condition. Each solution was then diluted 8 times to a concentration of 10^{-9}, then 0.1 ml of each dilution was inoculated to LAB selective media, MRS agar, supplemented with 0.5% CaCO3 in a petri dish and incubated at 37°C for 24-48 hours. The colonies that grew and formed a clear zone were then inoculated in the same agar to get a single colony. Each colony formed was then characterized on Gram and catalase and colonies with Gram positive and catalase negative were grown on MRS broth medium followed by stored as stock in 30% glycerol at -80ºC for further works. Colonies obtained from each fermented product were named as follows: GMBK for bekasam, GMCN for cincalok and GMKI for fish sauce.

Characterization of Lactic Acid Bacteria

Characterization of isolated lactic acid (LAB) bacteria including Gram staining test (using Gram staining kit (Sigma Aldrich)) and catalase test (using KOH 3%). According to Kusmawarti *et al*. (2014) a single isolate with Gram positive (+) and the catalase test (-) can be presumed as a LAB isolate.

Antibacterial Activity Test

1. Preparation of Histamine-Producing Bacteria (HPB)

The preparation of targeted histamine-producing bacteria (HPB) (*Morganella morganii* (TK7), *Klebsiella pneumoniae* (CK13.2), and *Citrobacter freundii* (CK1)), were done according to Farkas *et al*. (2018) by using optical density. All tested bacteria were incubated on TSB medium at 37ºC for 24 h. The OD_{600} were then adjusted to OD_{600} 0.1 by diluted in the same growth media and transfer to MRS broth media for antibacterial activity test. In the same time, the colony forming unit for each HPB was checked by total plate count (TPC) in TSB agar.

2. Preparation of Cell-Free Supernatant (CFS)

Preparation of cell-free supernatant (CFS) was performed according to Abrams *et al*. (2015). Lactic acid bacteria from glycerol stock were inoculated to MRS agar and grown at 37ºC for 24 h. The single colonies formed were then pre-cultured on MRS broth at 37ºC for 48 hours or until full growth (OD_{600} = 1). After incubation, pre-cultured LAB then re-inoculated (10% v/v) to a new 10 ml MRS broth media in a test tube and incubated at 37°C for 48 h. After 48 h, the bacterial culture was transferred to a centrifuge tube and centrifuged at 15,000 x g for 15 minutes to obtain the CFS. The CFS then transferred to a new sterile centrifuge tube,

heated at 100ºC for 3 minutes, measured the pH and neutralised with 0.1 N NaOH to eliminate the effect of the acid. To check whether the antibiotic activity came from bacteriocin like substances, the CFS was treated with protease (papain with final concentration 4 mg/ml) at its optimum enzyme activity (according to the maker) for 30 min

3. Cell-Free Supernatant (CFS) Antibacterial Activity Test on Histamine-producing Bacteria (HPB)

The inhibitory activities of the cell-free supernatant (CFS) on histamine-producing bacteria (HPB) were performed by macrodilution method according to Mariam *et al*. (2014) in a total volume of 5 ml. Cell-free supernatant in the proportion of 25% (v/v) of the final volume (1.25 ml) was added simultaneously with 1% (v/v) (50µl) HPB (OD_{600} 0.1). The sterilized MRSB in the same volume with CFS was used as negative control, while kanamycin (final concentration of 5 mg/ml) was used as positive control. All samples were then incubated at 37ºC for 24 h. The percent inhibitory activity (% inhibition) of the cell-free supernatant was obtained by measuring the decrease of the optical density (OD_{600}) on treated samples after incubation against negative controls.

RESULT AND DISCUSSION

Histamine-producing bacteria (HPB) are a group of bacteria that are able to produce histidine decarboxylase (HDC) which can convert free histidine to biogenic amine named histamine. HPB can be found in fresh fish as an indigenous bacterium or in fish products including fermented fish products caused by contamination. Our laboratory (Laboratory of Fisheries Product Quality and Safety Facilities) has successfully isolated several bacteria with decarboxylase activity from fresh fish including tuna and skipjack which were able to produce high histamine concentration in tuna infusion broth media (Wiranata, 2020). The histamine-producing isolates with high histamine production (more than 50 mg/kg) were continued to be identified by using 16s rRNA sequence-based technique and some of them (*Citrobacter freundii* CK1, *Morganella morganii* TK7, and *Klebsiella pneumoniae* CK13.2) were used in this research as a bacterial target. On the other hand, lactic acid bacteria are known to produce antibacterial substances named bacteriocin (Heredia-Castro *et al*, 2015; Abanoz and Buket, 2018; Simons *et al*., 2020). Therefore, the exploration of the potential of lactic acid bacteria to fight against HPB is expected to be able to provide a new solution to reduce the histamine content in fishery products especially fermented fish products.

Isolation of Lactic Acid Bacteria (LAB)

The initial isolation of targeted lactic acid bacteria (LAB) from bekasam, cincalok and fish sauce successfully obtained 22 isolates, 18 isolates and 17 isolates respectively. Further characterization using catalase test and Gram staining test suggested that only 12 isolates from bekasam, 10 from cincalok and 12 from fish sauce were strongly reassemble characteristics of LAB, including Gram positive, catalase negative (Nurhamidah *et al*., 2019) and showed same visual characteristics described by Corry *et al.* (2003) including color characteristic of white-yellowish to white-greyish color, and gives observable clear zone during inoculation on MRSA + 0.5% $CaCO_3$ (Figure 1). The clear zone as described by O'Bryan *et al*. (2015) is created by degradation of calcium carbonate by organic acid produced by LAB.

All putative LAB isolates were then inoculated to get a single colony, cultured in MRS broth and stored at -80oC in 30% glycerol for further analysis.

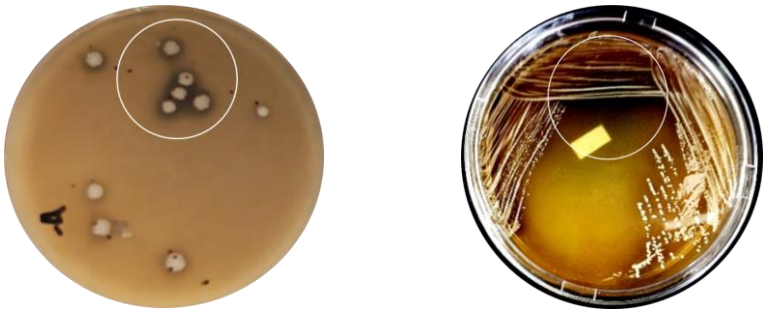

Figure 1. Targeted Lactic Acid Bacteria Formed a Clear Zone in MRS Agar Supplemented with 0.5% $CaCO_3$

Antibacterial Activity Test

All putative LABs from bekasam, cincalok, and fish sauce then tested for its antibacterial activity using macrodilution assay against *Citrobacter freundii* CK1, *Morganella morganii* TK7, and *Klebsiella pneumoniae* CK13.2. To obtain the cell free supernatant, all isolates were inoculated to MRS agar, followed by cultured in MRS broth. All isolates were reported to produce acid during incubation and lower the pH of MRS broth from near 7 to 4-5 at the end of incubation. The same phenomena were reported by Mariam *et al*. (2014); Wardani *et al*. (2017); and Rodríguez *et al*. (2019). To collect cell free supernatant, bacterial culture was centrifuged followed by heat treatment at 100ºC for 3 minutes. This step is designed to obtain antibacterial substances (targeted bacteriocin) which is susceptible to high heat treatment since this type of bacteriocin is very useful for industrial usage due to its resistance during high temperature process. After the heating step, the pH of cell free supernatants was adjusted to 7 to abolish the antibacterial activity due to acid. Prior to antibacterial activity tests by microdilution, total plate count (TPC) was conducted to calculate the colony forming unit (cfu) of used histamine-producing bacteria (HPB) at OD_{600} 0.1. The TPC showed that the average cfu of

HPB used was 1.47 x 107 cfu/ml. The antibacterial activity test by macrodilution was shown at Table 1

Lactic acid bacteria are reported to produce several antibacterial substances including hydrogen peroxide, organic acids and bacteriocin (Ban and Le, 2021). To check the possibility whether the antibacterial activity came from a bacteriocin-like substances, all isolates possessing antibacterial activity higher than 70% (GMCN 1.12, GMBK 2.6, GMBK 2.7, and GMKI 2.1) were then further treated with protease and again checked for antibacterial activity. The antibacterial activity after being treated with protease was depicted in Figure 2.

Table 1. Antibacterial Activity Against Histamine-Producing Bacteria

Samples*	% Inhibition against *Citrobacter freundii* CK1	% Inhibition against *Morganella morganii* TK7 (%)	% Inhibition against *Klebsiella pneumoniae* CK13.2 (%)
Positive control	100	100	100
Negative control	0	0	0
GMCN 1.12	88	44	50
GMCN 1.4	42	62	55
GMBK 1.10	51	61	18
GMBK 2.6	99	85	56
GMBK 2.7	99	98	84
GMKI 2.1	43	73	22

*Isolates with activity below 50% for all tested HPB were not shown; GMCN: isolates obtained from cincalok; GMBK: isolates obtained from bekasam; GMKI: isolates obtained from fish sauce; positive control: kanamycin (final conc. 5 mg/ml); negative control: sterile MRS broth

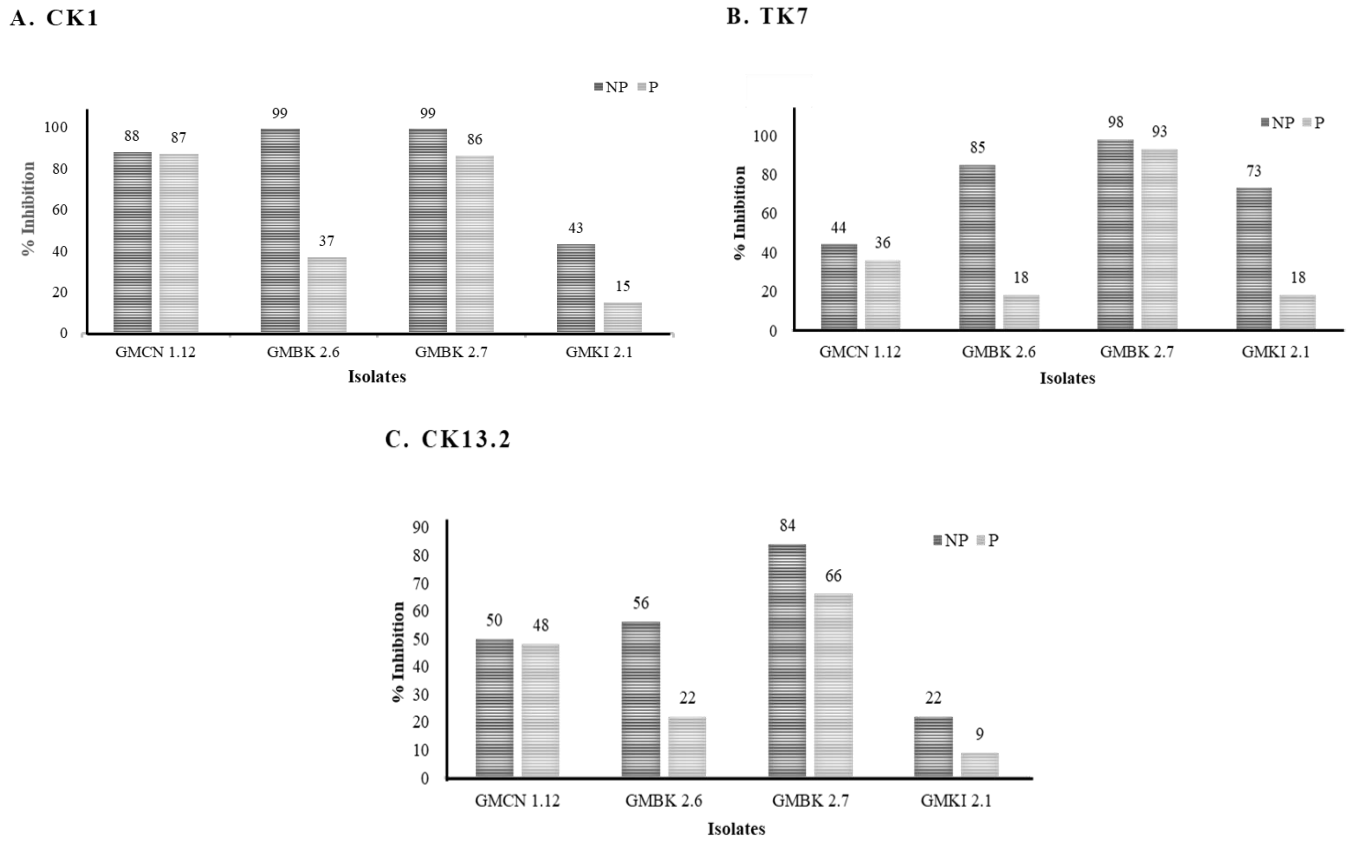

Figure 2. Antibacterial Activity After Treated with Protease (NP: non-protease; P: protease)

The addition of papain to CFS had an effect on decreasing the inhibitory activity in some samples (GMBK 2.6 and GMKI 2.1). The decrease of inhibitory activity could lead to the possibility that the antibacterial activity came from bacteriocin-like substances. Significant changes shown by CFS of GMBK 2.6, where the inhibitory activity against *Morganella morganii* (TK7) was decreased 67%, while the decrease of 62% and 34% were shown against *Citrobacter freundii* (CK1) and *Klebsiella pneumoniae* (CK13.2) respectively. These phenomena can be logically explained that proteases were able to cut peptide bonds of proteinaceous bacteriocin-like substances. The same results have been reported for example bacteriocin-like substance from *Pediococcus acidilactici* kp10 (Sidek *et al*., 2018); bacteriocin-like substance from *Pediococcus pentosaceus* (Azevedo *et al*., 2020); and bacteriocin-like substance from several probiotic bacteria (Hefzy *et al*., 2021) which also possess severe decrease or even lose the antibacterial activity after treated with protease.

However, the resistance of CFS against protease could not be excluded from the hypothesis that the antibacterial activity came from bacteriocin-like substances. The possibility is that the proteolytic enzyme used, has not been able to degrade the bacteriocin component so that it still has inhibitory effect against histamine-producing bacteria. Bacteriocin-like substance that resistance to proteolytic enzymes also occurred in several previous studies such as in Kusmarwati *et al*. (2014) who tested bacteriocin from lactic acid bacteria isolated from rusip products from Bangka and Kalimantan against 2 proteolytic enzymes. The bacteriocins obtained from the isolates of lactic acid bacteria RK1, RK2, RK4, and RA1 were tested using 2 proteolytic enzymes namely papain and proteinase-K, and gave the results that RK2 were resistant to papain enzymes and still gave inhibition to indicator bacteria but abolished afterward while treated with proteinase-K. In addition, research by Goh & Phillip (2015) stated that class II bacteriocins produced by *Weissella confusa* A3 were resistant to pepsin, proteinases, acidic pH, and heat.

Based on the antibacterial activity, GMBK 2.7 could become a potential candidate to be used as a starter in the fermentation process of fermented fish products. Further studies will be conducted in the near future regarding the bacterial identification, growth and antibacterial activity in high salt concentrations, preparation of bacterial active powder, and finally the application in the fermented fish process.

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CONCLUSION

From these results, we can conclude that in general, LAB isolated from bekasam, cincalok and fish sauce are having the ability to produce antibacterial substances and inhibit the growth of histamine-producing bacteria from low to high percent inhibition. The reduction in activity after treated with protease suggested that the antibacterial activity came prom proteinaceous like substance. The highest inhibition was shown by isolate GMBK 2.7, followed by GMBK 2.6, GMCN 1.12 and GMKI 2.1.

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