Production Of Antimicrobial And Bioactive Peptides From *Bakasam* Using *Enterococcus Faecium* 1.15 As A Starter

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Abstract.

The Development Of Traditional Fermented Food Products Into Functional Food, Utilizing Indigenous Microorganisms, Represents A Novel Innovation. Lactic Acid Bacteria (Lab), Which Are Abundantly And Readily Isolated From Native Fermented Foods, Hold Promise For The Exploration Of Local Lab Strains – A Strategic Step Towards The Development Of Probiotic-Based Foods In Indonesia. Enterococcus Faecium 1.15 Isolated From Bakasam Beef, Has Demonstrated The Capability To Produce Extracellular Proteases (4.83 U/Mg) And Displayed Inhibitory Effects Against Pathogenic Bacteria. The Production Of Bioactive Peptides, With A Size Less Than 30 Kda, Could Be Achieved Through Bakasam Fermentation Using E. Faecium 1.15 As A Starter For A Period Of 7 Days. This Study Promotes An Opportunity To Harness Local Microorganisms, Particularly Lab Strains In The Utilization Of Bioactive Peptides As Well As Enhancing The Nutritional Value Of Traditional Fermented Foods.

Keywords : *Bakasam, Lactic Acid Bacteria And Peptide Bioative.*

I. INTRODUCTION

Bioactive peptides are biologically active compounds that offer health benefits to the human body. They are derived through the enzymatic hydrolysis of proteins, including those from animal and plant sources, as well as via fermentation processes. Bioactives peptides are typically composed of 2 to 20 amino acids and exhibit various biological activities [1]. Among these, two types of bioactive peptides function as Angiotensin-Converting Enzyme (ACE) inhibitors, effectively preventing hypertension. These peptides were isolated from pork and produced through thermolysin dioxyme hydrolysis. The specific amino acid sequences of these biocative peptides are Met-Asn-Pro-Pro-Lys and Ile-Thr-Thr-Asn-Pro [2,3]. Furthermore, ham products also contain low-molecular-weight peptides with antioxidant and antihypertensive properties [4]. Processed meat products hold substantial promise for future development as functional foods [5]. Bioactive peptides can be isolated from pork using the hydrolysis process with papain, which employs enzymatic reactions. Alternatively, microorganisms, particularly Lactic Acid Bacteria (LAB), can be used as starters for food fermentation, further improving the production of these bioactive compounds [6]. Indonesia boasts numerous traditional fermentation products, incorporating both plant-based and animal-based proteins, representing local knowledge that offers great potential for deeper exploration. LAB play a crucial role in human life, primarily by participating in food fermentation and thriving within the intestinal environment.

These bacteria can be categorized into two groups: homo- and heterofermentative. Homofermentative LAB predominantly generate lactic acid, constituting roughly 90% of their metabolized products. In contrast, heterofermentative LAB produce lesser amount of lactic acid, with other byproducts e.g. acetic acid, ethanol, and CO_2 [7]. Examples of homofermentative LAB include S*treptococcus faecalis* and *Streptococcus liquifaciens*, while heterofermentative LAB encompass *Leuconostoc mesenteroides*, *Lactobacillus brevis*, and *Lactobacillus pentoaceticum*. Proteolytic enzymes from *Lactobacillus bulgaricus* were successfully isolated and purified using DEAE-cellulose chromatography and Sephadex G-150. The resulting enzyme, confirmed as a pure enzyme through synchronization with SDS-PAGE, displayed a molecular weight of 98,000 Daltons (Da). This enzyme exhibits optimal activity at pH 6.0, at 40°C. It

specifically targets the L-lysyl-4-nitroanilide substrate, and its activity is hindered by EDTA and 1,10-phenanthroline, while being activated by Ca^{2+} metal cofactors. Aminopeptidase obtained from *Streptococcus thermophilus*, was also isolated using DEAE-cellulose chromatography and Sephadex G-150. The purified enzyme possessed a molecular weight of 89,000 Da and functioned optimally at pH 6.5, at 35℃, and was activated by Mg^{2+} but inhibited by EDTA and 1,10-phenanthroline [8]. Furthermore, *Enterococcus faecium* 1.15, isolated from *Bakasam*, exhibits a notable proteolytic activity of 17.57 U/mL [9]. These indigenous LAB isolates, boasting proteolytic capabilities, can be employed as starters in the fermentation of meat products like Bakasam. Their role is to enzymatically hydrolyze the meat, ultimately yielding bioactive peptides.

LAB play a vital role in food fermentation, not only imparting distinc flavors but also extending the shelf life of products. This is attributed to their ability to produce metabolites that inhibit the growth of spoilage and pathogenic bacteria. The antimicrobial properties of LAB stem from the favorable conditions provided by the available nutrients, leading to competition with other bacteria, especially pathogens. LAB also produce lactic acid as the final product of sugar or carbohydrate metabolism. This lactic acid production lowers the pH of their environment, resulting in a sour taste. *Bakasam* is a traditional fermented meat product from Indonesia. During the *bakasam*-making process, sugar, salt and rice are added, and the anaerobic fermentation lasts for 7-14 days. Typically, no starter is employed in the *bakasam* fermentation process, leading to occasional spoilage by indoor microorganisms. Hence, this study introduced a starter: *Enterococcus faecium* 1.15, isolated from *bakasam*. The addition of this starter aims to enhance the fermentation process's efficiency and produce the desired bioactive peptides. In natural fermentation, all species/strains of bacteria and molds can grow without hindrance, impacting the pH value. The pH of fermented meat significantly drops after three days of fermentation. The high concentration of hydrogen ions (low pH) in meat has various effects on microbial growth, influencing the inhibition of spoilage microorganisms. Bioactive peptides from meat are obtained through enzymatic processes, often employing proteolytic enzymes like pepsin or proteases from plant or microbial sources. The development of bioactive peptide-based meat production through fermentation, utilizing indigenous LAB isolates with potential probiotic activity, presents a promising avenue for further research.

II. MATERIALS AND METHODS

2.1 Fermentation of *Bakasam*

The preparation of *bakasam* involved the following ingredients: 50 g of beef shank, 5 g of salt, 10 g of rice, and 5% starter. Fresh beef shank was coated with salt, left for 15 min, rice was then added, and the mixture was stirred thoroughly. Subsequently, the starter was inoculated, and the mixture was placed into a sealed container. Fermentation was carried out at 37 ℃ for 7 days. Sampling was performed to analyze the Total Plate Count (TPC) in colony-forming units per gram (cfu/g), which assessed the viable count of LAB and proteolytic LAB.

2.2 Isolation of Lactic Acid Bacteria

Bakasam samples (1 g) were minced and placed in a microtube containing 1 mL of 0.85% NaCl. The samples were diluted following the standard serial dilution procedure until 10^{-6}, then an aliquot of 100 µL was inoculated and spread evenly onto a plate containing MRS Agar medium supplemented with 3% (w/v) of skim milk powder. The plates were incubated for 24 hours at 37 °C. After incubation, the number of growing LAB colonies was counted, reflecting the population of indigenous LAB.

2.3 Growth Profile of Lactic Acid Bacteria

The growth of *Enterococcus faecium* 1.15 was observed in MRS Broth medium at 37°C, under static condition in a batch system. An aliquot (1 mL) of broth culture was sampled for every 5 hours and measured for its optical density at 600 nm (OD_{600}) to estimate the bacterial growth.

2.4 Antagonistic Test of LAB against Spoilage and Pathogenic Bacteria

Pseudomonas cocovenenans, *Salmonella* sp., *Staphylococcus aureus* were cultivated in LB medium, and aseptically spread on MRS Agar medium with a concentration of 1×10^8 CFU/mL. Four agar plugs (⌀ = 60 mm) from active-growing *E. faecium* 1.15 colony were placed proportionally on top of bacterial lawn

then the plates were incubated at 37ºC overnight. Positive results were achieved when clear zones measured in millimeters (mm), appeared around agar plugs.

2.5 Proteolytic Activity Assay

Proteolytic activity was analyzed quantitatively using casein as a substrate. The protease activity test was carried out using the Enyard method [10]. This test was carried out using a 96 well microplate. A total of 18 µl of reaction mixture consisting of 6 µl of sample, 6 µl of Tris buffer and 6 µl of substrate was incubated for 30 minutes at 37 ºC. The enzymatic reaction was stopped by adding 12 µl trichloroacetic acid (TCA). Tyrosine solutions were used as standards ranging from 0, 25, 50, 100, 125, 250, 500, and 1000 mM. A total of 143 µl of working reagent (Na_2CO_3) and $CuSO_4.5H_2O$ with a ratio of 5:1) and 30 µl of Folin Ciocalteau reagent were added to the reaction mixture, then centrifuged for 5 minutes at a speed of 10,000 g. The absorbance of free tyrosine in the supernatant was measured at 620 nm and the concentration was compared using a tyrosine standard curve. One unit of proteolytic activity is defined as the amount of sample that releases 1 µmol of tyrosine per minute under experimental conditions.

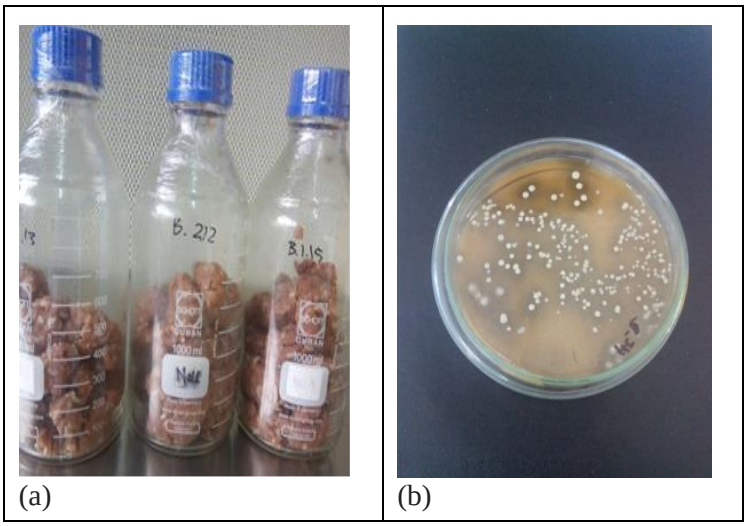
2.6 Sodium Dodecyl Sulphate – Polyacrylamide Gel Electrophoresis (SDS-PAGE) Analysis

The molecular weight of bioactive peptides produced by *E. faecium* 1.15 was determined using SDS-PAGE technique. The crude peptides were recovered from an optimized growth of *E. faecium* culture through a series of ammonium sulphate precipitation. Molecular weight analysis was carried out using SDS PAGE brand Novex-Tris Glysin. 15 µL sample and 10 µL loading dye were mixed and heated at ± 95 ºC for 10 minutes to denature the protein. The denatured sample was inserted into the gel well and then the electrophoresis machine was run at 110 volts for 1 hour. After protein separation was complete, the gel was removed and staining was carried out using Simplyblue. Next, destaining using distilled water and then documented. The Precision Plus Protein Dual Xtra Standard protein marker (2-250 kD) is used to predict the size of the protein bands that appear.

III. RESULT AND DISCUSSION

3.1 *Bakasam*

Bakasam is a traditional fermented product that has the potential to be developed as a functional food. *Bakasam* can be produced from various types of meat, including rabbit, lamb, duck, chicken and beef. The results of *bakasam* fermentation using different meat types are presented in Table 1. *Bakasam* from beef exhibited a low pH (4.25) and the highest LAB count (2.22×10^4 cfu/g). All LAB isolates were chosen based on their proteolytic activity as evident from a clear zone around the colony. The resulting Bakasam is depicted in Fig. 1 (a), while LAB isolates with proteolytic activity are shown in Fig. 1 (b).



(a) (b)

Fig 1. Bakasam from fermented beef (a) and LAB isolate from Bakasam with proteolytic activity (clear zone) (b)

The pH value and total number of lactic acid bacteria from various types of bakasam with different types of meat are presented in Table.1.

Table 1. Characterization of bakasam with various meat

Bakasam (Source)	pH	Viable count of LAB (cfu/g)
Rabbit	4.96	1.11×10^{4}
Lamb	4.78	1.00×10^{4}
Duck	4.67	1.05×10^{4}
Chicken	4.68	1.23×10^{4}
Beef	4.25	2.22×10^{4}

Processed meat products hold significant potential for future development as functional foods [11]. Bioactive peptides isolated from pork through papain hydrolysis can be produced using enzymatic reactions or by employing microorganisms, specifically LAB, as starters in food fermentation. Indonesia boasts numerous traditional fermented products based on both vegetable and animal proteins, representing a local wisdom that offers further exploration opportunities. LAB play a crucial role in human life, both in the context of food fermentation and its presence in the intestinal microbiota.

3.2 Isolation and Screening of Lactic Acid Bacteria

LAB isolation from *bakasam* prepared with various types of meat using MRSA-specific media showed promise as a probiotic source. Through further isolation and purification, a single potential LAB isolate, namely LAB 1.15, was successfully obtained. The potential of this LAB isolate to inhibit the growth of pathogenic bacteria is presented in Table 1. When compared to the antibiotic control, the inhibitory capacity of LAB 1.15 is relatively lower. However, it retains significant potential since additional purification steps may lead to the production of bioactive peptides with enhanced antimicrobial properties.

Table 2. Colony inhibition by LAB isolates from *bakasam* against spoilage and pathogenic bacteria

LAB isolates	Diameter of inhibitory zones (cm)		
	Pseudomonas cocovenenans	*Salmonella* sp.	*Staphylococcus aureus*
Isolate 1.15	1.64	1.80	1.31
Ampicillin	3.24	2.11	3.13

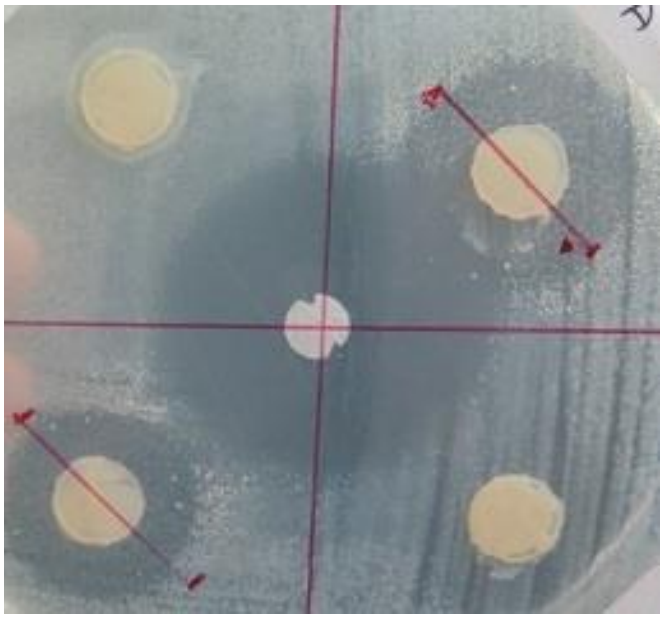


Fig 2. Inhibition of isolate.1.15 with phatogenic bacteria

Lactic Acid Bacteria has an important role in human life, both through its involvement in food fermentation and its ability to grow on the path of intestine. Lactic acid bacteria in general can be divided into two kinds of homofermentatif and heterofermentatif. In the homofermentative class the largest fermentation result is lactic acid which is approximately 90%, whereas in heterofermentative the amount of lactic acid produced is less than 90% or approximately equal to other products such as acetic acid, ethanol, CO2 [7]. Lactic acid bacteria belong to homofermentatives such as Streptococcus faecalis and *Streptococcus liquifaciens*, while those including heterofermentative eg *Leuconostoc mesenteroides*, *Lactobacillus brevis*, and *Lactobacillus pentoaceticum* [7]. Lactic acid bacteria in the fermentation of food in addition to providing a distinctive flavor, this bacteria also extend durability due to its ability to produce metabolite products that can inhibit the growth of bacterial decay and bacterial pathogens.

3.3 Growth Curve of *Enterococcos faecium* 1.15

LAB isolates selected 1.15 that have been identified molecularly 99% identical to *Enterococcus faecium* are potential LAB isolates as probiotics. Inhibitory resistance of these isolates to some pathogenic bacteria showed a considerable activity. Information relating to growth curve *E faecium* is expected to provide basic information related to the characteristics of the isolate and is required for its further utilization.

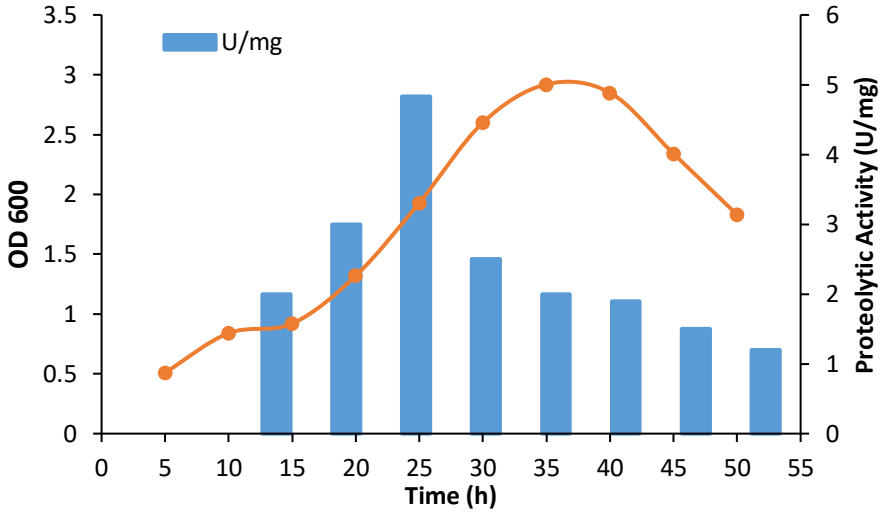


Fig 3. Growth curve of *E. faecium* 1.15 at temperature 37 ^{0}C

The growth of *E faecium* 1.15 is optimum at 37˚C in MRS broth and produces extracellular proteases and metabolite peptides at the 40th hour in the stationary phase of the bacterial growth phase. At the 25th hour of growth, the highest protease enzyme was excreted with a specific activity of 4.83 U/mg. Meanwhile, the crude extract protease produced by *L plantarum* 1.13 isolate isolated from Bakasam had an activity of 2.93 U/mg [8].

3.4 *Bakasam* Fermentation Using *Enterococcus faecium* 1.15 as starter

Bakasam, a traditional fermented meat product, holds potential as a functional food. This study has identified promising LAB isolates, particularly *E. faecium* 1.15, known for their strong antimicrobial activity. Introducing LAB isolates as starters in *bakasam* fermentation is anticipated to enhance product flavor and yield bioactive peptides with desired properties. The inclusion of the probiotic starter, *E. faecium* 1.15 resulted in a noteworthy increase in the total LAB count and proteolytic LAB over the 7-day *bakasam* fermentation. This augmentation in LAB population within *bakasam* should lead to higher bioactive peptide production. The elevated levels of biomass and proteolytic LAB cells correspond with an increase in extracellular protease enzyme production, which, in turn, should enhance the breakdown of meat proteins into bioactive peptide fragments. Furthermore, the rise in the overall LAB count (inclusive of both proteolytic and non-proteolytic strains) is expected to augment the potential of *bakasam* as a functional food with probiotic attributes.

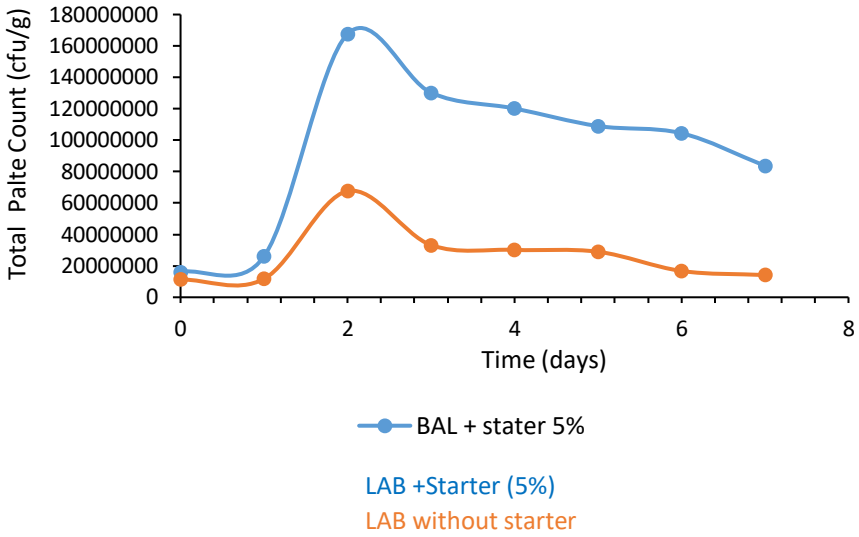


Fig 4. Comparison of amount of proteolytic LAB and LAB between *Bakasam* with the addition of a 5% starter *Enterococcus faecium* 1.15 and *Bakasam* without the addition of starter (native)

The observations were conducted on *bakasam* with various fermentation durations ranging from the first day until seven days. The parameters assessed included the viable count of LAB with proteolytic activity, the pH of the *bakasam* products, as well as the presence of bioactive peptides in crudeextracts and their antimicrobial activity. The introduction of indigenous LAB, specifically *E. faecium* 1.15 isolates, substantially increased the total LAB count and the number of LAB with proteolytic activity compared to *bakasam* without starter. On the second day of fermentation, *bakasam* with the *E. faecium* 1.15 starter exhibited a significant rise, with a total count of 1.6×10^8 cfu/g compared to the one without starter (6.7×10^7 cfu/g). From days 2 to 7 of fermentation, there was a decrease in the total LAB count. This reduction can be attributed to competition between the added starter and the indigenous LAB originating from the meat.

3.5 SDS-PAGE

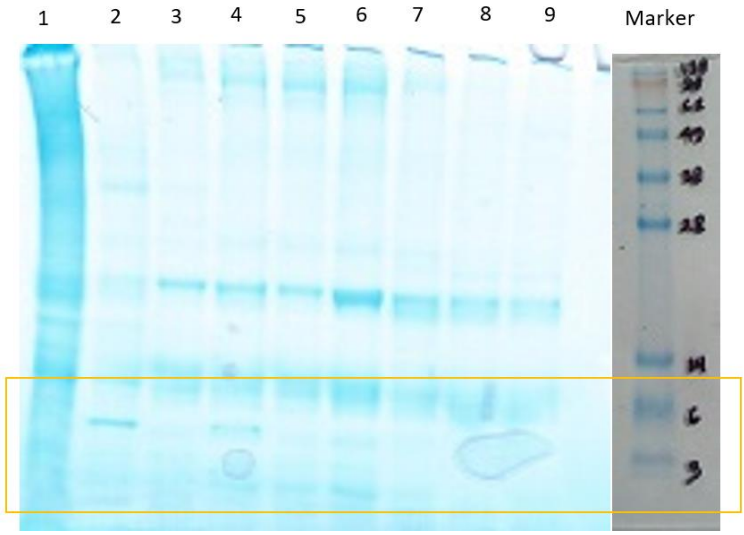


Fig 5. Profile SDS-PAGE crude peptida bioaktif from bakasam with starter *Enterococcus faecium* 1.15. Beef extract (lane 1), crude peptide with ammonium sulfat 60% presipitation (lane 2), crude peptide bakasam days 1-7 (lane 3-9).

To determine whether *bakasam* fermentation with the addition of *E. faecium* 1.15 is capable of producing bioactive peptides, an SDS-PAGE analysis was conducted on days 1-7 of fermentation. The results of the analysis revealed the presence of small peptides, each measuring less than 30 kDa. The production of bioactive peptides through fermentation utilizing a local LAB starter exhibits significant developmental potential. In *katsubushi* fermentation, for instance, using Aspergillus sydowii, bioactive peptides in the range of 0.2 – 17 kDa are produced [12].

IV. CONCLUSION

In conclusion, the LAB isolate, *Enterococcus faecium* 1.15, which was isolated from *bakasam* beef, possesses the capacity to produce extracellular protease enzymes with a specific activity of 4.83 U/mg, along with inhibitory effects against pathogenic bacteria. The production of bioactive peptides can be achieved through the fermentation of *bakasam* products with the inclusion of the *E. faecium* 1.15 for a duration of 7 days.

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