Enterococcus faecium 1.15 ISOLATED FROM BAKASAM SHOWED MILK CLOTTING ACTIVITY

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Abstract

Rennin-like protease of many microorganisms behave similarly to chymosin and are potential alternatives of rennet. The lactic acid bacteria with milk clotting activity were isolated from Bakasam, an Indonesian traditional fermented meat. Screening assay was carried out using modified method of skim milk agar and milk clotting activity test, and the isolate was then identified using 16S rRNA. We found 4 isolates that showed MCA of 18-20 SU/mL. Identification using 16S rRNA indicated that the isolate ALG.1.15 was 99% identical with *Enterococcus faecium.* The isolate potentially produced renin-like protease to subtitute renin from veal.

Keywords: *lactic acid bacteria, milk clotting activity, rennin-like protease*

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Introduction

Rennin-like protease has remained attractions for research and exploration, and development of this protease is potential in Indonesia. Milk-clotting microbial enzymes are meaningful for substitution of calf rennet in the cheese industry. Microbial rennet is more desirable for some reasons including less expensive, greater biochemical diversity, and easier genetic modification. Milk Clotting enzymes play an important role in the process of cheese maturation (Kumar *et al*. 2005). Rennet is a crude protease an animal-derived crude protease which curdles milk and plays an important role in cheese maturation (El-Tanboly *et al*. 2013).

Rennet from calves used in cheese production is increasingly high prices campared with calf rennet so the need for a suitable rennet substitutes is rapidly becoming acute for various sources (Shah *et al*. 2014).

Some microbes have been reported to have the ability in the production of protease that has similar characteristics to rennin produced from calf stomach. Thus, the protease is called as a rennin-like protease. The protease-producing microorganisms included *Rhizomucor miehei*, *Rhizomucor pusillus*, *Endothia parasitica*, *Aspergillus oryzae*, and *Irpex lacteus* (Jacob *et al*. 2011). The crude enzyme extract produced by *Bacillus stearothermophilus* exhibited high milk-clotting activity (MCA) of 24,23 U/mL, while *Bacillus subtilis* MTCC 10422 exhibits milk curd forming capacity (Narwal *et al*. 2016).

Enterococci are lactic acid bacteria found in food of animal origin (milk, cheese, meat, and fermented sausages), and are responsible for ripening process of cheese and formation of flavors in meat and dairy products (Abriouel *et al*. 2005). The purpose of this study is to isolate strains of rennin-like producing lactic acid bacteria for substituting the conventional rennin.

Materials and Methods

Fermentation of Bakasam

Bakasam was a traditional fermented food from Lampung, Indonesia. The ingredients of Bakasam were 20 gram of rice, 2 gram of salt, 0.2 gram of sugar, and 100 gram of top side meat. The fermentation was carried out until 15 days under un-aerobic condition.

Preparation of samples and isolation of LAB with Proteolytic Activity

Lactic acid bacteria were isolated from Bakasam with various raw meats (beef, lamb, chicken, rabbit, and duck). Isolation process was using MRSA oxoid selective media with skim 3%.

Proteolytic Activity Test on Skim Milk Agar

Isolates that had a high activity of extracellular proteolytic enzyme showed a clear zone surrounding colonies. Isolates that showed width clear zone were selected for quantitative proteolytic activity test.

Milk-Clotting Activity Test

To determine the enzyme activity in milk coagulation, MCA used casein (in $CaCl_2$) as substrate incubated at 35 °C for 5 min. Rennin extract was then added. The milk clotting activity (MCA) of the enzyme was measured by the method described by El-Taboly et al. (2013). A 5 mL of the substrate (12% skim milk in 10 mM $CaCl_2$) was incubated for 5 min at 35°C and then 0.5 mL of enzyme extract was added. Time required for formation of the first particles was recorded, and the milk-clotting activity was calculated according to Kawai and Mukai (1970) as follows: SU = 2400×5×D/T×0.5 (1), where T is milk-clotting time (s), and D is dilution of the enzyme. One Soxhlet Unit (SU) of milk-clotting activity was defined as the amount of enzyme required to clot 1 mL of substrate in 40 min at 35°C.

DNA Extraction

LAB isolates (two isolates with milk clotting activity) were cultured in MRS broth (pH 7.0) at 37^0C for 1 day. Bacterial cells were collected by centrifugation at 6.000 rpm for 10 min. The genomic DNA was extracted as previously described, with modification (Zhu 1993). The pellet was resuspended with TE buffer (10 mM Tris HCL pH 8, 1 mM EDTA), 40 µL of lysozyme (60 mg/mL), and incubated at 37 °C for 60 min. A total of 200 µl 10% SDS, 100 µL 5 M NaCl, 80 µL 10% CATB were added, and warmed at 68 °C for 30 min, and an equal amount of chloroform was added. The mixture was centrifuged at 13.000 rpm for 10 min. The supernatan was collected and added with ethanol (PA). The mixture was shaken again and then centrifuged at 13,000 rpm for 10 min. After being air-dried, the DNA was dissolved in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) and the concentration was adjusted to 10 µg/mL DNA and stored at -20^0C for further analysis.

PCR Amplification for 16S rRNA gene

For 16S rRNA gene sequencing, primers 8F (5'AGAGTTTGATCATGG CTC AG-3'; positions 8 to 27 bp) and 15R (5'- AAGGAG GTG ATC CAA CCG CA-3'; positions 1541 to 1522 bp) were used to amplify partial length gene of bacterial 16SrRNA fragment (Cho *et al*.2008). Each 25µl of PCR mixture contained 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 M $MgCl_2$, 200 µM of each dNTP, 400 nM of each primer, 1 U of Taq polymerase, and 10 ng of DNA tamplate. The PCR was operated at 96 °C for 5 min, and performed 35 cycles consisting of 96 °C for 1 min, 58 °C for 3 min, and 72 °C for 1 min; and 72°C for 7 min. The PCR products were subjected to gel electrophoresis in 1% agarose gel, followed by ethidium bromide staining.

DNA Sequencing and phylogenetic Analysis

The DNA sequencing was performed in Malaysia (Genetica science company). Similarity searches with sequences were performed by online BLAST analysis in NCBI. For phylogenetic analysis, sequences were aligned by using the CLUSTAL X software (Thompson, 1997).

Results

LABs isolation and screening

Isolation of LAB from Bakasam used MRSA media containings 3% of skim. Isolates obtained exhibited a clear zone around the colonies, and the isolates were then purified. We found that the isolates morfology showed cocci shapes with an average diameter of 1.69 µm.

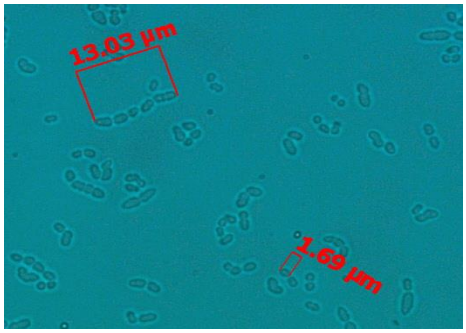

Figure 1. Morphology of *Enterococcus faecium* isolated from Bakasam

Screening on Skim Milk Agar

Some researchers have used casein substrate for screening protease-producing microbes (Verma *et al*.2001; Chi *et al*.2007; Sindhu *et al*, 2009).

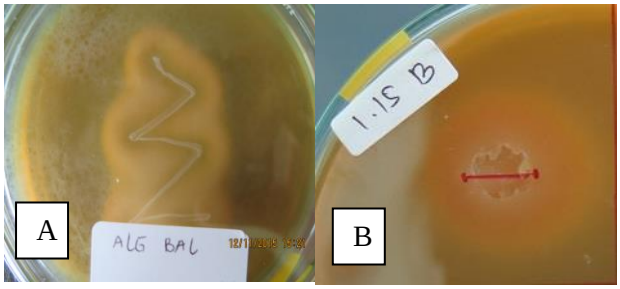

Figure 2. Skim Milk Agar Test :(A) Colonies of isolate ALG.1.15 at MRSA media + skim 3% ; (B) Colonies of isolate at Block Agar MRSA media + skim 3% for quantification of coltting zone diameter (cm)

LAB Isolates with clotting activity exhibited the formation of casein clotting around the colony as shown in Figure 2. To obtain quantitative analysis of casein coagulation, further experiment was carried out using modified screening method as prescribed by Wendry *et al*. (2015). We found that 4 of 30 isolates showed casein coagulation with different diameters formed around the colony (Figure 2). Differences in diameter represented the ability of the isolates in producing extracellular proteases which are able to clot casein.

Casein coagulation or clotting is result of the presence of acid and rennin enzyme. Enzymatic coagulation of milk was caused by the loss of the peptide bond between phenylalanine and methionine (the sequence to the 105 to 106) of the polypeptide kappa casein, leading to the formation of para kappa casein (para k-casein) and macropeptides (Suhartono 1989; Fox *et al*. 2000). Macropeptides produced from this process were soluble in water, but k-casein will be precipitated. Instability of casein micelle was associated with interaction of Phe and Met, thus other casein fractions were formed.

Table 1. Isolate characteristics on skim milk agar

No.	Isolates	Clear Zone (cm)	Clotting Zone (cm)
1	ALG.1.12	-	1.5
2	ALG.1.13	-	1.4
3	ALG.1.14	-	1.5
4	ALG.1.15	-	2.1

Clotting zone is associated with the ability of the isolates to excrete rennin-like protease. Our results exhibited that ALG.1.15 isolate resulted in the greatest clotting zone (Table 1).

However, excessive proteolytic activity also may cause high hydrolysis on casein which is responsible for reduction of casein clotting (curd). Consequently, this leads to production of hydrophobic peptides that contribute to bitter taste of the cheese (Sausa *et al*. 2001).

Milk Clotting Activity

Casein coagulation or clotting occurs because the lactic acid and the enzyme is rennin. Enzymatic coagulation of milk was caused by the loss of the peptide bond between phenylalanine and methionine (the sequence of 105 to 106) of the kappa casein polypeptides, resulting in para k-casein and macropeptides (Suhartono 1989; Fox *et al*. 2000). The length of time required since the enzyme extract is added to the casein coagulation showed enzyme activity (Ottani *et al*. 1991).

Figure 3. Milk clotting activity of crude extract of rennin-like protease *Enterococcus faecium*.

Microbial rennin was produced by *Bacillus subtilis*, *Rhizomucor pusillus*, *R. miehei*, *Endothia parasitica*, *Aspergillus oryzae*, *Bacillus sphaericus*. MCA of *Mucor pusillus* was 50 SU/mL (crude enzyme) and 100 SU/mL (NH_4SO_4 (40-80%) (El - Tanboly *et al*. 2013). Our results showed that ALG.1.15 isolate resulted in the highest MCA (20 SU/mL) as presented in Table 2.

Table 2. Milk clotting activity of *E faecium* isolate

No.	Isolates	Milk Clotting Activity (SU/mL)
1	ALG.1.12	18
2	ALG.1.13	18
3	ALG.1.14	18
4	ALG.1.15a and 1.15 b	20

Identification of Lactic Acid Bacteria

The partial 16S rRNA gene sequences (1.500 bp) of two isolates with highest milk clotting activity (ALG.1.15a and 1.15 b) were determined. Then, the sequences were compared with related bacteria in GenBank and sequence similarities were determined using the BLAST program.

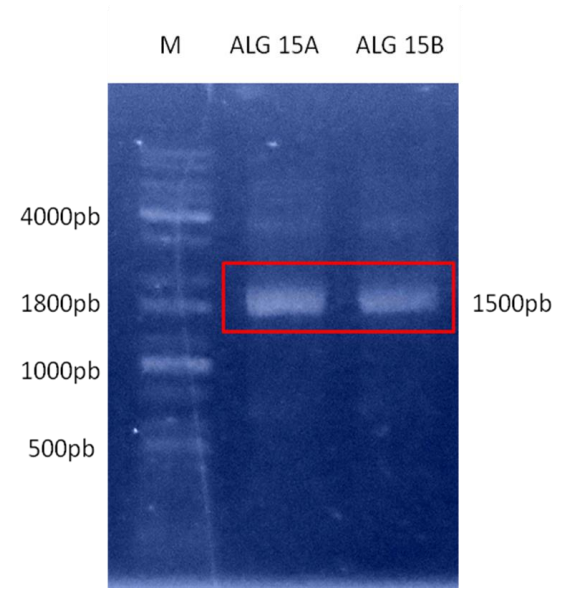

Figure.4. The result of PCR amplification from Bakasam isolates.

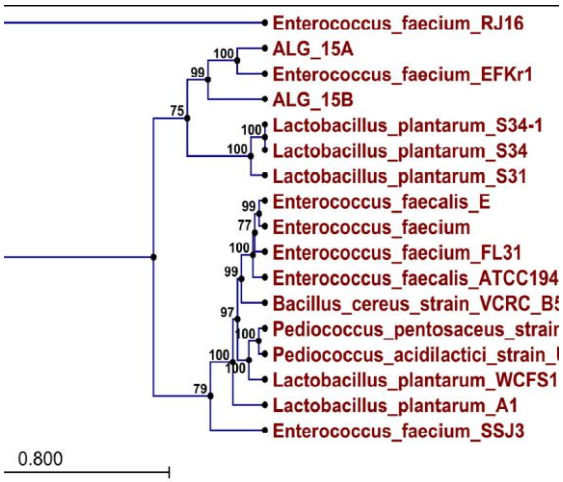

Figure.5. Phylogenic tree of analyzed sequence by using BLAST

The result showed that Bakasam isolates of ALG.1.15a and ALG.1.15b were 99% identical with *Enterococcus faecium.*

Discussion

It was reported that the number of LAB in Bakasam meat during fermentation process increased until the fourth day, but its pH decreased. After that, their number gradually decreased. The final number of LAB in Bakasam meat was 1.67 x 10^6 cfu/gram (Roostita *et al*. 2010). Identification using 16S rRNA showed that LAB in Bakasam was

from *Lactobacillus plantarum* strains (Mustopa *et al*.2014). The extracellular proteases secreted by *L. plantarum* S31 have been isolated and purified from supernatant of 20-day cultivation (Budiarto *et al*. 2016).

LAB as "friendly bacteria" have a great potential in the development of biotechnology-based food industry. LAB have the ability to produce proteolytic enzymes around the cell wall, membrane, the cytoplasm within the cell. For instance, *Lactobacillus acidophilus* has the capability of generating extracellular protease.

Food processing industry in Indonesia still heavily depends on the importing protease, inhibiting the development of the livestock-based food industry. Renin is an enzyme required in the manufacture of cheese, responsible for curdling process or casein coagulation. Currently, the rennin was not only produced from calf stomach, but also from microbes. Rennin from animal origin (calf stomach) is accounted for 30-40% of rennin production worldwide. Rennin (EC.3.4.23) is an acidic protease, and able to obtain from the stomach (abomasum) of calves (veal) (Suhartono, 1989; Kloosterman, 1991).

Rennin could be combined with acid, but the combination showed different results of clotting activity. Furthermore, milk is not only coagulated by rennin, but also by proteases from plant sources (such as papain and bromelain) and microorganisms. However, the use of protease from plant sources has remained great challenge due to excessive proteolytic activity formed by these proteases.

Some microorganisms have produced protease that showed similar properties to rennin produced from calf stomach. Therefore, the protease was acknowledged as rennin-like protease. *Rhizomucor miehei*, *Rhizomucor pusillus*, *Endothia parasitica*, *Aspergillus oryzae*, and *Irpex lacteus* been produced commercially for producing rennin-like protease (Jacob *et al*. 2011).

It could be concluded that *Enterococus faecium* ALG.1.15 from Bakasam showed an ability to clot milk with MCA 20 SU / mL, necessitating further purification efforts to increase its activity.

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