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Antimicrobial Properties and *Lactase* Activities from Selected Probiotic *Lactobacillus brevis* Associated With Green Cacao Fermentation in West Sumatra, Indonesia

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Abstract

Lactase activity is very important enzyme for Lactose Intolerant people. The lactic acid bacteria selected for use as Probiotic bacteria should be able to tolerate many organotoxic or heavy metal toxicities in intestine at least for 90 minute, attach to the epithelium, and grow in the lower intestinal tract before they can start providing any health benefits. The Green varieties of cacao fruits were used for cacao bean fermentation. The cacao bean covers with white pulp containing fructose, e.g. 42 mg/g, glucose 24 mg/g and sucrose 21 mg/g, as carbon source of Lactic Acid Bacteria (LAB). Screening of LAB was carried out after 24-36 h during spontaneous fermentation. This paper aims to find potential LAB resistant acid pH and producing potential antimicrobial and showing high protease/lactase activities. The medium of de Man, Ragosa, and Sharpe (MRS) were used to screen LAB, and 63 colonies were found. The screening of isolates is based on LAB survival growth in acid pH ranges (2.0; 2.5 and 3.0) and body temperature (37°C). For Antimicrobial experiments were used pathogen bacterial *E. coli* and *Salmonela* (Unand Collection) as indicator strain. Six isolate were conformed as strong antimicrobial and selected as potential for producing protease (lactase) using specific Triple Sugar Iron Agar (TSIA) medium. The isolate G3 and G6 were selected for further enzyme protease delivered with 2% Skim Milk as a protein subsrate. The results showed, the maximum amount of protease activity potential from isolate G6 in acid pH (3.0) were calculated as 0.0088 and protease activity 1.1795 Unit/mL, while the optimum of protease activity was found at pH (6.0) calculated protease activity 3.150 Unit/mL. This study could explain the possibility of using potential isolate G6 LAB as Probiotic for dairy or food Industry and supplement tablet. There is no report so far concerning this topic and potential isolate G6, with high antimicrobial or wide pH of protease. The study will continue to purify antimicrobial Bacteriocin and amino acid structure determination. The G6 isolate was 95% polymorphism with *Lactobacillus brevis*.

Keywords: Antimicrobial; *Lactobacillus brevis*; Protease, *Theobroma cacao Linn*

Introduction

Cacao fermentation is important for improvement of chocolate flavor, food industries, and nutrition. The protease enzyme from lactic acid bacteria (LAB) is thought to play a vital role because amino acids resulting from proteolysis are the major precursors of specific flavor compounds, such as various alcohols, aldehydes, acids, esters and sulfur compounds [1,2]. The pH after 3 days of cacao fermentation will down from pH 5.0 to pH 3.0, where concentration of lactic acid increased from 0.3 (mg/g) to 5.0 (mg/g) were reported [1,3]. The interesting of LAB when they produce strong antimicrobial activity against microbes including food spoilage organism and pathogen by producing various compounds such as organic acids, diacetyl, hydrogen peroxide and bacteriocins or bacterial peptides during lactic acid fermentation. Lactic Acid Bacteria are known for their Probiotic properties are consider as 'food grade' microbes, and generally recognized as Safe (GRAS), used extensively in food industries and human nutrition. Many reports of Lactic Acid bacteria benefit of intestinal health and the immune system, as well as anti-carcinogenic, anti-diarrheal and hypocholesterolaemic effects, improve lactose utilization [4,5]. As many as 75 percent of African and Native Americans and 90 percent of Asian-Americans are lactose intolerant. In this case, the lactic acid bacteria selected for use as Probiotic bacteria should be able to tolerated acid environment at least for 90 minute, attach to the epithelium, and grow in the lower intestinal tract before they can start providing any health benefits [6,7]. Probiotic bacteria should be characterized in acid shock response which is varying among bacterial species. The acid isolate that can grow at pH 3.5 and also has the ability to grow in neutral pH is the potential to characterize the protease and lactose activities under acid and neutral pH [7,8].

There are two properties which allow bacteria to metabolize lactose, firstly they must have an intracellular transport system allowing them to take up the lactose and secondly they have to be able to produce the enzyme β-galactosidase (also known as Lactase). This enzyme breaks down lactose into β-galactose and glucose, which can then be metabolized to produce energy. The specificities of protease also play an essential role to degrade of unexpected compounds such as bitter peptides; therefore this development will impact the organoleptic quality of the food fermented products [2,9]. This is also true to benefit for cacao bean fermentation which is value added for farmer and improves organoleptic quality in chocolate industries [2].

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Proteolytic activity of *Lactobacillus* has been associated with cell-wall-bound proteinases [10,11]. Most of LAB were reported and isolated from high protein sources such as animal milk, cheese, and yoghourt, but very view report LAB have been isolated from acid environment of fermented tropical fruits and protease biochemically studied. There is no publication concerning screening and invitro antimicrobial of LAB and characterizing its protease potential during spontaneous cacao fermentation. This paper aims to study potential antimicrobial acid tolerant LAB and protease activities during Green Cacao fermentation in West Sumatra. Strain selected acid tolerant and potential antimicrobial is important to the next further study of antimicrobial Bacteriocin of amino acid structure, physiology and molecular DNA of LAB for new Probiotic and application in food preservative [5,12].

Materials and Methods

The present study was done by experiments where determination of antimicrobial activity of Lactic Acid bacteria conducted more than triplicates.

Bacterial isolation [13]

Lactic Acid Bacteria was isolated from the bean pulp of cacao Green after 36 h fermentation. Strains were culture on MRS Agar or in MRS broth at 37°C for 48 h in anaerobic jar. Dilution method (10^{-1}–10^{-8}), of sample were prepared and plated on de Man Rogosa (MRS) agar medium. Stock cultures were prepared by growing the strain for 24 h at 37°C, and were preserved in vials with glycerol 20% v/v, at - 70°C. The colonies were used for selection in acid condition (the screening of isolates based on LAB survival growth in acid pH ranges (2.0; 2.5 and 3.0) and body temperature about 37°C. Purify colony repeated plating and studied for their Gram reaction, cell and colony morphology, catalase-negative, cocci or rod shape isolates with characteristic and considered as lactic acid bacteria.

Screening growth cultures [9,14]

Each strain from the stock was grown in MRS broth medium. The medium were autoclaved at 121°C/15 min. cells were harvested by centrifugation (4500xg for 10 min at 4°C), washed three times in sterile saline (0.85% NaCl), inoculated into MRS broth acidified with concentrated hydrochloric acid to pH ranges (2.0; 2.5 and 3.0), or nonacidified MRS broth (pH 6.0 and 7.0) and incubated at 37°C for 24 h. Before and after incubation, plate counts were done by pour plate technique. The growing strains were consider being candidates for selection of acid-tolerant strains and were used to isolate potential acid tolerant colony. The acids-tolerant candidate was further investigated for screening lactase using specific Triple Sugar Iron Agar (TSIA) medium [15] and protease activity [9]. TSIA medium containing glucose, lactose and sucrose with phenol red and FeSO4 acts as indicators. The growth of LAB were done by inoculated sterile medium with 2% (v/v) of the activated cultures and incubated at 37°C for 0, 12, 24, 36 and 48 hrs and LAB was total colony count. The experiments were determined at intervals for 12 hrs.

Antimicrobial assay [10,12,16]

The antimicrobial spectrum from LAB was determined using well diffusion method. A loopful of each of the LAB isolates from the MRS agar slants was inoculated into tubes containing 10 mL of sterile MRS broth. These broth cultures were incubated at 37°C for 48 h. After incubation, the cultures were centrifuged (5000 rpm for 35 min at 4°C) to obtain the Culture Free Supernatant (CFS). The pH of the CFSs was adjusted to pH 7 with 1 M NaOH to exclude antimicrobial effects of organic acids. Control for each tube was prepared using un-inoculated MRS broth. Sterile cotton swabs were dipped into the cultures of the test (indicator) microorganisms (previously propagated in Brain Heart Infusion (BHI) broth for 24 h at 37°C) and inoculated by swabbing over the entire surface of the pre-set Mueller-Hinton agar plates. Care was taken to evenly distribute the test pathogens bacteria such as E.coli and Salmonella, throughout the entire surface of the plates. The bacterial chossen for indicator based on intestinal common bacterias. Sterile filter paper discs of 5 mm diameter were prepared from Whatman No. 1 filter paper. Each disc was impregnated with the respective culture supernatant, air dried and placed on a 150 mm plate, within 5 to 15 min after swabbing the test pathogens. After 18 to 24 h of incubation at 37°C each plate was examined for the zone of inhibition. The diameters of the inhibitory zones were measured including the diameters of the discs to the nearest whole number.

Identification of lactic acid bacteria [13]

Identification of the selected isolates (with the desired antimicrobial activity) was carried out using morphological and biochemical methods. The identification of the isolates was performed. The studies included motility, catalase test, Gram's staining, cell morphology, and carbohydrate fermentation [3,13].

PH value

The pH value of media was measured using pH meter, combined with glass electrode (Beckman 40). Values of pH of LAB grown in MRS broth at 37°C were measured at 24 hrs in acid and neutral pH.

Determination of protein content [17]

Protein content was determined by using colorimetric at maximum absorption at 600 nm, using brilliant blue G-250 and Bovine Serum Albumin (BSA).

Protease activity determination [10]

Protease activity of culture supernatant was determined using casein as substrate. One ml of substrate (1% casein in 0.005 M phosphate buffer, pH 7.0) was incubated at 37°C for 15 min, and then 1.0 ml of the culture supernatant which was obtained by centrifugation (8000xg at 4°C for 20 min) was added. After mixing, the reaction mixture was incubated at 37°C for 20 min. The reaction was terminated by adding 2.0 ml of 0.4 M trichloroacetic acids (TCA) then filtrated and the mixture was further incubated at the same temperature for 20 min. For the blank, the substrate was precipitated with TCA before adding the enzyme solution and then treated as follow: to one ml of the filtrate obtained after TCA precipitation, 5.0 ml of 0.4 M sodium carbonate solution was added followed by 1.0 ml of Folin reagent and incubated at 37°C for 20 min for color development and reading absorbance (A) at 750 nm. A unit of protease activity is defined as the amount of enzyme required to release TCA- soluble fragment giving a blue color equivalent to one µg of tyrosine under the same condition of the assay.

Protease specific activity [17]

Protease specific activity was calculated from dividing the determined protease activity values on the results of protein content.

Results and Discussion

Isolation and identification of lactic acid bacteria

Cacao bean fermentation was performed in woody box with several

holes and the length of (40 cm) x the breadth (40 cm). The average pH after 36 hours fermentation down to pH 3, 0 and homogenized pulp was taken for LAB isolation. Six colonies were selected from 63 colonies showing in Figure 1A, and two potential resistant acid colonies (G3 and G6) were morphology or biochemically studied. Two isolates (G3 and G6) were identification and explain in Table 1. The resistant acid isolates were round colonies, creamy, smooth, catalase negative and heterofermentative. Several carbohydrates such as glucose, fructose, sucrose and maltose can be used for medium fermentation.

Diversity of Lactic acid bacteria, including *Lactobacillus brevis* also found in Nigeria during cacao fermentation was reported [2]. Our isolates were further study for their protease biochemical properties. The total colony of potential LAB (G3 and G6) as shown in Table 2. Produced high Total colony in acid pH (2.5) during 36 hours fermentation (27×108) Cfu/mL. These results also explain by other report, concerning the possibility of acid resistant colony of *Lactobacillus acidophilus* to grow in intestinal tract for 3 days long [18]. Several resistant acid of *Lactobacillus acidophilus* also can grow better in acid pH (3.5), but only 90 min observation.

Lactic acid bacteria with diffrent spesies that can growth in acid pH (3.0) were also reported [5,10], during 24 and 36 hours of fermentation. The acid resistant bacteria are useful for producing small peptide or amino acid and precursor for flavor development during cheese and milk fermentation. Screening extracellular protease as showing in Figure 1B, could explain the protease G6 isolate produced higher clear zone when compare to G3 isolate in NA medium containing 2% skim milk at pH (3.0). These results explain that the G6 isolate is concerned as potential having higher protease activity using 2% skim milk as a substrate, and resistant acid pH with clear zone diameter of (17 mm) and G3 only (8 mm). Other investigation suggest that bacterial isolate resistant acid pH is potential to select and useful for biochemical study of protease for application in food industries such as yoghurt, cheese and others or human/animal health [3,5].

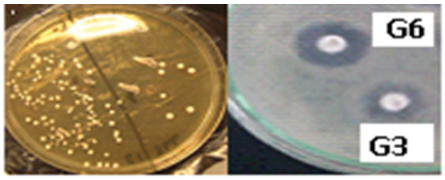

Figure 1: Purification of colony LAB (A); Screening Protease isolate of G6 and G3 in NA medium containing 2% skim milk, pH 3.0, 37°C for 24 hours (B).

Biochemical Characteristic	Results
Colony morphology	Creamy, smooth, round colonies
Gram staining	Gram positive, Cocus and Rod
Growth in MRS broth	Uniform turbidity
Type of fermentation	Heterofermentative
Growth in acid PH	Resistant
Catalase	Negative
Growth in fruc, suc, maltose	positive

Table 1: The biochemical characteristic of selected LAB of (G3 and G6) potential Colonies.

Isolate	grow at 12 (h)	grow at 18 (h)	grow at 24 (h)	grow at 36 (h)
G3	5×10^4	25×10^5	5×10^6	25×10^7
G6	15×10^5	25×10^6	25×10^7	27×10^8

Table 2: Total colony of selected LAB grow in acid pH (2.5) at 370C, with different time of incubation.

Pathogen	pH 2.0	pH 2.5	pH 3.0	pH 6.0	pH 7.0
E. coli	14.2 ± 0.35	14.5 ± 0.70	15.0 ± 0.00	25.8 ± 0.35	24.5 ± 0.70
Salmonela	14.0 ± 0.00	15.0 ± 0.00	14.5 ± 0.70	25.5± 0.35	24.5 ± 0.70
Amp (30 µl)	20 ± 0.00	21 ± 0.00	21.5 ± 0.00	24.0 ± 0.00	23.8 ± 0.35

Table 3: Average of diameter inhibition zone (mm) and antimicrobial Activity of culture Supernatant of G6 isolate (50 µl) against pathogens at 370C, pH acid and neutral for 24 h, (Mean ± SD).

In vitro antimicrobial characterization of G6 and G3 isolate was shown in Table 3. The results shows that the G6 isolate (50 µl) having strong antimicrobial Bacteriocin as compare than control antibiotic ampicillin (30 µl). Every microorganism has a minimal, a maximal and an optimal pH for growth and metabolism [12]. Microbial cells are significantly affected by the pH of their immediate environment, thus studying the effect of acid pH on the antimicrobial compounds and protease produced by our isolates was an important criterion for application of Probiotic for intestinal tract or human and animal health and food preservative [8,10]. The results obtained in our study regarding the resistant acid pH still capability of having high invitro antimicrobial properties.

According to other report, antimicrobial proteinaceous such as Bacteriocin produce by bacteria varying greatly in chemical structure, mode of action and specificity [5,12]. Antibacterial produce by Lactic acid bacteria provided successful results or application in health also in food industries were reported [3,13]. The G6 resistant acid also has wide range of pH for Antimicrobial properties as compare to antibiotic Ampicillin. This isolate could be important, since the isolates have the ability to survive, grow and produce their antimicrobials both under acidic and alkaline conditions as reported [8,19]. This results confirm that G6 isolates not only resistant to acid pH also produce antimicrobial which is important application during cacao bean fermentation, can killed pathogen bacteria's and produce flavor or specific aromas. Recently, report explain that *Lactobacillus* casei NRRL B1922, has optimum acid pH at 3.60 and high proteolytic activity in cheese industry. Other reported explain the important of acid protease LAB to degrade peptide and produce aromas in dairy industries [3,11]. The protease specific activity showing in Table 4, was optimum protease producing and specific activity in pH 6.0 (0.0210) and for protease activity was found 3.150 (unit/mL). In Table 3, the Inhibition zone of acid resistant G6 isolate in pH (2.0, 2.5 and 3.0) showing middle

Protease Specific Activity	Protease Activity (Unit/mL)
0.015	0.335
0.058	0.855
0.088	1.979
0.210	3.755
0.176	2.910

P<0.05

Table 4: Protease specific activity and protease activity (Unit/mL) of Isolate G6, grown in MRS broth at 37°C, 24 hours, at different pH.

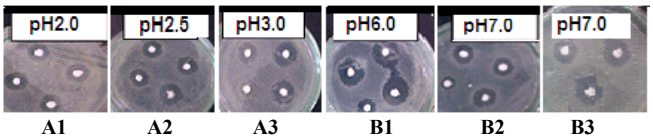

Figure 2: The average of antimicrobial activity of potential isolate G6 against *E.coli* pathogen showing Diameter clear zone; Inhibition: (A1, A2, A3) in Acid pH (2.0; 2.5 and 3.0) And (B1, B2) in Neutral pH (6.0 and 7.0); Control ampicllin in pH 7.0 (B3).

antimicrobial as compare to Ampicillin, but in pH 6 the G6 isolate showing the highest antimicrobial properties more than 25 (mm). The maximum antimicrobial activity was pH 6.0, while in pH 7.0, showing a little decrease. In Figure 2, showing the action of G6 isolate, for inhibition zone (mm) both in acid and neutral pH. In Table 3 (pH 3.0, We observed protease specific activity was increase start from pH 2.0, and 2.5 more than 6 times in pH (3.0). So far the Acidic proteoliytic Enzyme is valuable enzyme for digestion in Human, Anmal and dairy industries [8,18].

This protease enzyme might be different chemical structure, active site and potential for purification and structure elucidation. Increase protease activity in acid pH and were found novel extracellular serine protease in *Lactobacillus paracasei* was reported [11].

The maximum protease activity was also found as much as 3.755 (Unit/mL) during 24 hours of dairy fermentation [19]. In this report our protease activity concern as higher protease activity to recent report. This investigation will continue to study for purification of protease enzyme and antimicrobial Bacteriocin to study the amino acid structure and peptide degradation.

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References

1. Ardhana, M Made (2003) The Microbial Ecology of Cocoa Bean Fermentations in Indonesia Int J Food Microbiol 86: 87-99.
2. Smit G, Smit BA, Engels WJ (2005) Flavour formation by lactic acid bacteria and biochemical flavour profiling of cheese products, FEMS Microbiol Rev 29: 591-610.
3. Sumaryati Syukur (2010) Fermentation of Cacao Bean, Specific Cacao Aroma and Potential LAB Isolated from West Sumatra Environment. Proceedings International Seminar of Agriculture, University of Andalas, Padang, Indonesia.
4. Gibson GR, Probert HM, Van Loo J, Rastall RA (2004) Dietary modulation of the Human colonic microbiota: updating the concept of prebiotics. Nut Res Rev 17: 259-275.
5. Sumaryati Syukur and Endang Purwati (2013) Biotechnology Probiotic. ISBN: 978-979-29-3998-9.
6. Yasushi Kawai, Kiyoshi Tadokoro, Ryotaro Konomi, Kazumi Itoh, Tadao Saito, et al. (1999) A novel method for the detection of protease and the development of extracellular protease in early growth stage of *Lactobacillus* delbrueckii. J Dairy Sci 82: 481-485.
7. Macfarlane GT, Allison C, Gibson GR (2008) Effect of pH on protease activities in the large intestine. Letters in Applied Microbiology 7: 161-164.
8. Yañez Francisco JC, Ramon Pacheco A, Fernando Luis GC, Marı´ a de LA Navarrete-Del Toro (2003) Characterization of Acidic Proteolytic Enzyme from Monterey Sardine (Sardinops sagax caerulea) viscera. Journal of Food Chemistry 85: 343-350.
9. Broadbent JR, M Barnes, C Brennand, M Strickland, K Houck, et al. (2002) Contribution of Lactococcus lactis cell envelope proteinase specificity to peptide accumulation and bitterness in reduced-fat Cheddar cheese, Appl Environ Microbiol 68: 1778-1785.
10. AM Kholif, GA Mohron, MA El-Nawawy, Azza A Ismail, MME Salem, et al. (2011) Evaluation of Proteolitic Activity of Some Dairy Lactobacilli. World Journal of Dairy & Food Sciences 6: 21-26.
11. Wang SL, Wang CW, Huang TY (2007) Microbial reclamation of squid pen for the production of a novel extracellular protease by Lactobasillus paracasei subsp paracasei TKU012. Bioresour Technol 99: 3411-3417.
12. Ahn C, ME Stiles (1990) Antibacterial activity of lactic acid bacteria isolated from vacuum-packaged meats. Journal of Applied Bacteriology 69: 302-310.
13. De Man, Rogossa JC, MS Elisabeth (1960) A Medium for Cultivation of Lactobacilli. Journal of Applied Bacteriology 23: 130-135.
14. Dave RL, Shah NP (2006) Evaluation of media for selective enumeration of *Lactobacillus* delbrueckii, *Lactobacillus acidophilus* and Bifidobacteria. Journal of Dairy Science 79: 1529-1536.
15. Patrick R Murray, Elien Jo Baron, Michael P faller, Tenover (1999) Manual of clinical microbiology. (7thedn), ASM Press, USA.
16. Gianella RA (1994) Importance of the intestinal inflammatory reaction in Salmonella-mediated intestinal secretion. Infect Immun 23: 140-145.
17. Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Annual Biochemistry 72: 248-254.
18. Vélez MP (2007) Identification and Characterization of Starter Lactic Acid Bacteria and Probiotics from Columbian dairy Products. Journal of Applied Microbiology 103: 666-674.
19. Chou LS, Weimer B (1999) Isolation and Characterization Acid and Bile Tolerant Isolate from Strains of *Lactobacillus acidophilus*. J Dairy Sci 82: 23-31.

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