ISSN: 0975-8585

Research Journal of Pharmaceutical, Biological and Chemical Sciences

Isolation, Antimicrobial Activity and Protein Bacteriocin Characterization of Lactic Acid Bacteria Isolated from Dadih in Solok, West Sumatera, Indonesia.

Sumaryati Syukur[1]*, Edy Fachrial[1], and Jamsari[2].

[1]Laboratory of Biotechnology, Department of Chemistry, Faculty of Mathematics and Natural Sciences, Andalas University, Padang, 25163, Indonesia.
[2]Laboratory of Biotechnology and Plant Breeding, Department of Agroecotechnology, Faculty of Agriculture, Andalas University, Padang, 25613, Indonesia.

ABSTRACT

Dadih is West Sumatera traditional food made by natural fermentation of buffalo milk in bamboo which contains some lactic acid bacteria (LAB) that produce some antimicrobial substance such as organic acid, diacetyl, hydrogen peroxide and bacteriocin or bactericidal protein. These substance especially bacteriocin have ability to inhibit the growth of food spoilage bacteria and some bacteriocin are stable in high temperature. This makes LAB can be used as an ideal biopreservatives because in some food processing need high temperature. Total colony was 84 x 10^8 CFU/ml. Six colonies that produce clear zone in medium was selected for further purification. The colonies then characterized biochemically by Gram's staining, catalase test, fermentation type .The antimicrobial activity of neutralized pH supernatant (pH=6) only give positive result on isolate E2 and E3 The best isolate then exposed by heat at 80^{o}C, 90^{o}C and 100^{o}C and then the antimicrobial activity against pathogenic bacteria was measured. The best and stable antimicrobial activity was shown by isolate E2 (±12mm against *E.coli*, ±12 mm against *S.aureus* and ±13mm against *S.typhi*). The extracellular protein or crude bacteriocin of isolate E2 precipitated by using ammonium sulphate precipitation. The protein content was determined by Bradford method and the protein concentration was 1.45mg/ml. The molecular weight of crude bacteriocin was measured using Tris Glycine SDS PAGE. The molecular weight of crude bacteriocin was below 10kDA, indicate that bacteriocin was class II bacteriocin that stable with high temperature. This is the first time we reported bacteriocin protein from those dadih.

Keyword : Dadih, Lactic Acid Bacteria, Antimicrobial Activity, Bacteriocin

Corresponding author

ISSN: 0975-8585

INTRODUCTION

Lactic Acid Bacteria (LAB) can be isolated from different source such as from fermented fish product, Budu [1], freshwater fish [2] fermented milk products [3,4,5], raw goat's milk [6], dried fruits [7]. LAB are a group of gram-positive bacteria, cocci or rods form and non spore forming They produce lactic acid as the major end product during the fermentation of carbohydrates. It consisted of many genus including *Aerococcus, Carnobacterium, Enterococcus, Lactobacillus, Lactococcus, Leuconostoc, Pediococcus, Streptococcus, Tetragenococcus*, *Vagococcus* and *Weisella* [8].

LAB have traditionally been associated with food fermentations and generally considered as a beneficial microorganism generally considered as beneficial microorganisms [9]. They can produce several compounds that contribute to taste, smell, color, and texture of the foods. They also can produce some antimicrobial substances such as organic acids, diacetyl, hydrogen peroxide, and bactericidal protein or bacteriocins. These substances have ability to inhibit pathogenic and food spoilage bacteria . Lactic acid bacteria commonly used as a natural food preservative in order to improve the food safety and stability [10]. Bacteriocins are antimicrobial peptide produced by bacteria to inhibit the growth or kill other bacteria and effective against Gram positive bacteria. Many LAB produce bacteriocins and have been shown to be safe and have potential as an effective natural food preservatives . Bacteriocins produced by LAB could be subdivided into four classes. Class I, the lantibiotics (containing lanthionine) a small and heat stable peptides, and usually consists of 19-39 amino acid in length, class II, a small, non lanthionine containing and heat stable (usually < 10kDa) class III, a large molecules bacteriocinc and sensitive to heat (30-300kDa) and class IV, a complex bacteriocin that require carbohydrate or lipid moieties [11].

Dadih is West Sumatera traditional foodfrom West Sumatera, Indonesia, made by natural fermentation of buffalo milk in bamboo which contains some Lactic Acid Bacteria (LAB) [12]. This study aimed to screen on potential LAB that posses antagonism activity against a few selected Gram positive and Gram negative bacteria and estimating the molecular weight of bacteriocin produced by LAB which isolated from *dadih*

MATERIALS AND METHODS

Experimental

Total colony was counted by using Colony Counter. Inhibition zone was measured by vernier caliper. Growth phase was determined by spectrophotometer. Molecular size of protein was determined by SDS PAGE with 17% concentration separating gel, power supply was adjust on 200V.

LAB isolation, enumeration and biochemical characteristic

Dadih samples (1 g) were homogenized with 9 ml MRS broth in test tube and incubated 24 h at 37^{o}C in anaerobic condition. Isolation of bacteria was carried out on de-Mann-Rogosa-Sharpe (MRS) agar from serial dilutions (10^{-1} to 10^{-8}) using pepton water supplemented with 1,5% $CaCO_3$.0,1 ml aliquots of the appropriate dilutions were surfaced-plated on MRS agar, incubated anaerobically at 37^{o}C for 48h. Six colonies that showed a clear area around the surface of agar plate were purified by repeated streaking onto MRS agar. The following characteristic were investigated for each isolate : Gram staining, growth phase, catalase test and fermentation type.

Activity of isolated lactic acid bacteria on pathogen bacteria

Antimicrobial activity of LAB against *E.coli*, *S. aureus*, and *S. typhi* was determined using disc diffusion assay. Six single isolated colonies were selected from MRS agar plates and transferred to grow in sterile MRS broth.The broth culture was incubated anaerobically at 37^{o}C for 24h. The indicator microorganism (*E.coli, S.aureus, S.thypi*) were grown in Nutrient Broth at 37^{o}C for 24h. Using sterile cotton swab, the indicator microorganism swabbed into the surface of MRS Agar. The sterile paper disc (6mm) dipped into LAB culture and into sterile MRS broth as negative control, and put onto the surface of swabbed MRS agar. After 24h incubation, each plate then evaluated and diameters of inhibition zone including diameter of the discs then measured.

ISSN: 0975-8585

Activity of cell free supernatant on pathogen bacteria

The isolated LAB which possessing the best activity were selected and grown in MRS broth for 18h to 24 h at 37^{o}C and cell free supernatant (CFS) obtained by centrifugation at 10.000 rpm for 30 minutes at 4^{o}C. The supernatant then collected and some supernatant neutralized with NaOH 3N with the aid of filter strips in order to eliminate the effect of organic acid. The supernatant which were un neutralized and neutralized then used for antibacterial activity using disc diffusion assay. After 24 h incubation diameters of inhibition zone including diameter of the discs then measured

Activity of heat treated cell free supernatant (CFS)

Sensitivity to heat was tested by heating the CFS to 60^{o}C, 80^{o}C and 100^{o}C for 15 minutes, then residual activity was checked by disc diffusion assay.

Estimation of protein and molecular size determination by SDS-PAGE

The protein content of *Lactobacillus* sp was estimated using Bradford method, and the partial purification was done by ammonium sulphate precipitation. The CFS were mixed with different ammonium sulphate concentration 60% and 70%. This mixture was allowed to stand overnight at 4^{o}C.The samples was centrifuged at 14.000 rpm for 15 min, the the pellet was collected and dissolved in 0.1M potassium phosphate buffer. The above samples were taken for molecular size determination using SDS-PAGE and the protein fragment compared with protein marker

RESULT AND DISCUSSION

LAB isolation, enumeration and biochemical characteristic

One sample of dadih was used for isolation of lactic acid bacteria and 84 x 10^{8}CFU/ml were obtained from 1 g of dadih. 6 isolates LAB that produce clear zone around colony were picked for the next treatment. The clear zone appearance is due to the dissolution of $CaCO_3$ in MRS medium by acid agent. The 6 LAB isolates purified by re-streaking on MRS agar. All the six isolates were Gram positive, catalase negative and homofermentative. From biochemical characteristics and Gram staining the LAB were identified to *Lactobacillus* sp. The result for biochemical characteristic shown in **Table.1** .The growth phase of LAB isolates shown in **Figure 1.**

Table 1: Biochemical characteristic of LAB isolated from dadih

LAB isolates	Biochemical characteristic			
	Gram staining	Catalase test	Fermentation type	Colony morphology
E1	Positive	Negative	Homofermentative	Creamy, smooth,round colonies
E2	Positve	Negative	Homofermentative	Creamy, smooth,round colonies
E3	Positive	Negative	Homofermentative	Creamy, smooth,round colonies
E4	Positve	Negative	Homofermentative	Creamy, smooth,round colonies
E5	Positive	Negative	Homofermentative	Creamy, smooth,round colonies
E6	Positive	Negative	Homofermentative	Creamy, smooth,round colonies

LAB have been associated with food and feed fermentation and are generally considered as a beneficial microorganism. LAB constitutes a group of Gram positive bacteria, aerotolerant cocci or rod and lack genuine catalase [9]. The diversity of LAB from milk also found in Iran. LAB have been isolated from Ewe milk in Myaneh and Hashrood, Iran, 77 out of 168 bacterial colonies from 63 samples Ewe milk, yoghurt and sour buttermilk were identified belonging to genus LAB based on their biochemical characteristic such as Gram reaction, catalase test and morphology. LAB are genus of bacteria which were catalase negative and Gram positive [13].

ISSN: 0975-8585

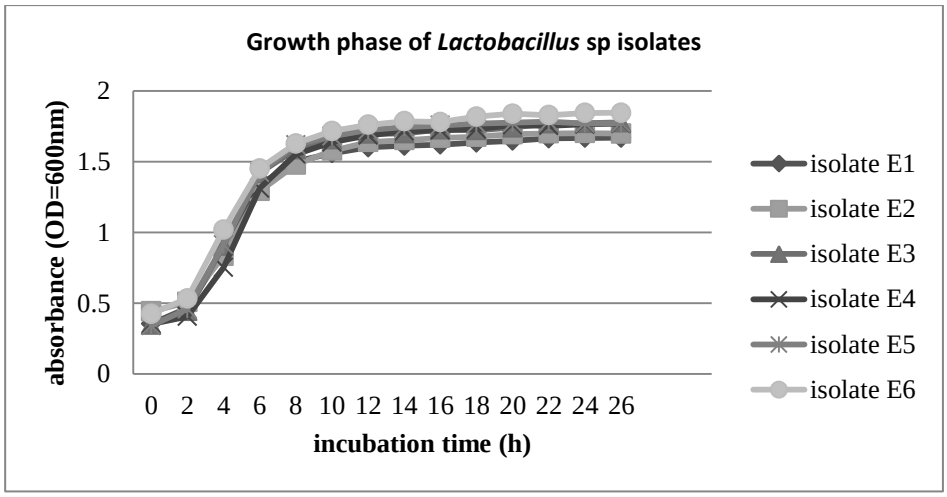


Figure 1: Growth phase of *Lactobacillus* sp isolates

Activity of isolated lactic acid bacteria on pathogen bacteria

The inhibition zone of LAB culture against pathogen bacteria shown in **Figure 2**. The diameter inhibition zone against *S.typhi* were between 10.5mm-14mm (include diameter of disc = 6mm) where the strongest inhibition zone was on E1 and the weakest was on E4. The strongest inhibition zone against *S.aureus* was on E4 (14mm) and the weakest was on E1 (12.5mm). The strongest inhibition zone against *E.coli* was on E1 (12mm) and the weakest was on E3 and E5 (10.5mm). Generally *S.aureus* was more sensitive against LAB compared with *E.coli* or *S. typhi.*

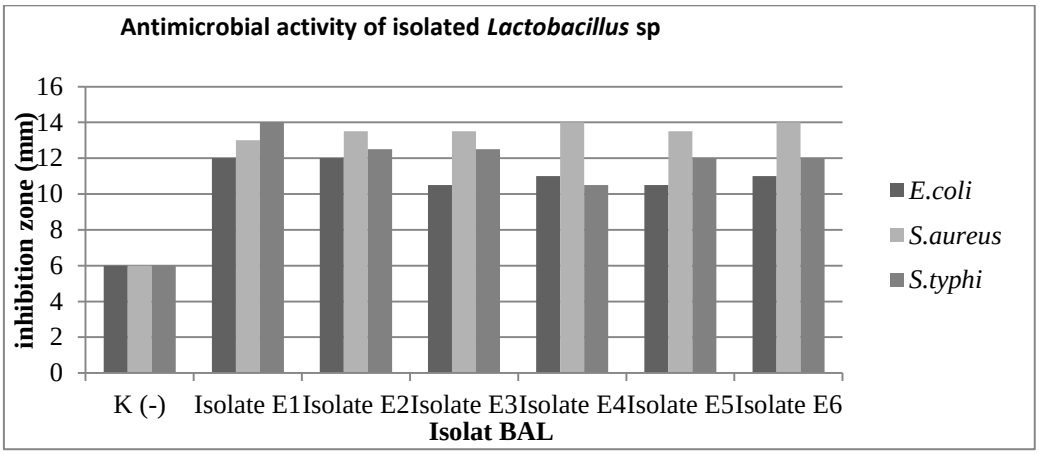


Figure 2: Antimicrobial activity of isolated *Lactobacillus* sp against pathogen bacteria

In the other report, in Cameroon LAB isolated from “pendidam” a local fermented milk product and assayed their antimicrobial activity against *E.coli* and *S.aureus* . 20 isolates were selected and and all the isolates can inhibit the growth of *S.aureus* with zone of diameter between 1mm-4mm, and 14 isolates out of 20 isolates showed inhibition against *E.coli* [14]. Antimicrobial mechanism of LAB including organics acid production, hydrogen peroxide, diacetyl and bacteriocin. The effect of antimicrobial directly given by organic acids including lactic acid, acetic and propionic that believed from the action of the acids on the bacterial cytoplasmic membrane which interfere with the maintenance of membrane potential and inhibit active transport [15].

Activity of cell free supernatant (CFS) on pathogen bacteria

The activity of pH un-neutralized CFS against pathogen bacteria shown in **Figure 3**. The highest inhibition zone was shown against *S.aureus* on isolate E3 (13.4mm) followed by isolate E2 (12.6mm). Inhibition

ISSN: 0975-8585

zone against E.coli was between 9mm-11mm and the strongest activity was shown by isolate E2 and E3 (11mm). Inhibition zone against S.typhi was between 9.2mm-10.4mm with the strongest activity was shown by E2 (10.4mm) and the weakest activity was on E5 (9.2mm).

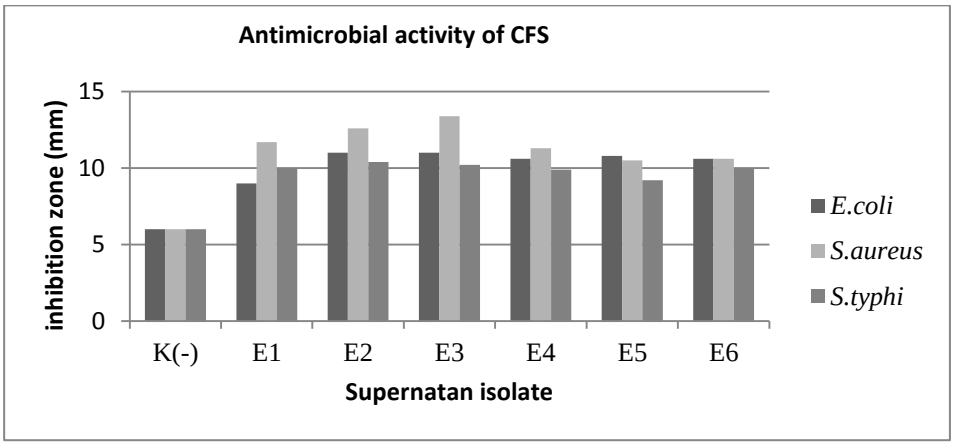


Figure 3: Antimicrobial Activity of Cell Free Supernatant (CFS) against pathogen bacteria

The antimicrobial effect exerted by LAB might be caused by production of lactic acid, reduction of pH, diacetyl and hydrogen peroxide and other primary and secondary antimicrobial metabolites such as bacteriocin [16]. Askari *et al* [7] have reported that LAB isolated from dried fruits have a high sensitivity against Gram positive bacteria. Bacteriocin are not frequently active against Gram negative bacteria, because the outer membrane of this bacteria acts as a permeability barrier for the cell to prevent the molecules such as antibiotics, detergents and dyes to reach cytoplasmic membrane [17]. The inhibition zone of unneutralised CFS against pathogen bacteria shown in **Figure 4**

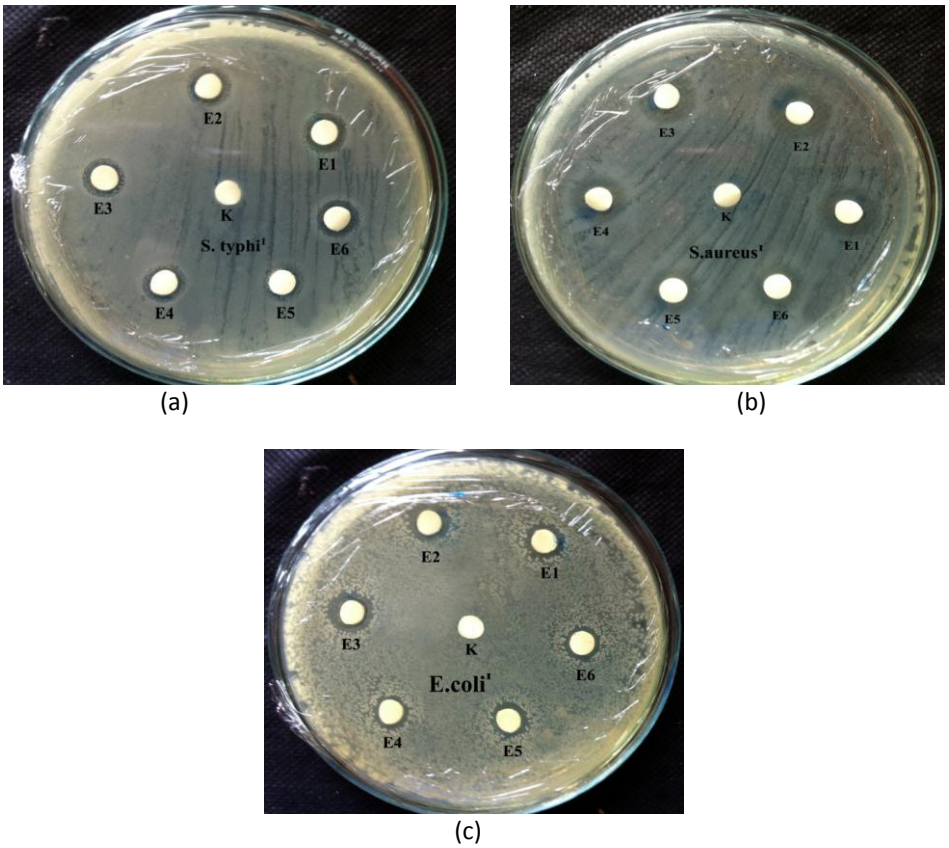


Figure 4: inhibition zone of unneutralised CFS against pathogen bacteria : (a) CFS against *S.typhi* (b) CFS against *S.aureus* (c) CFS against *E.coli*

ISSN: 0975-8585

Figure 5 shows only isolates E2 and E3 still have antimicrobial activity, while isolated E1, E4, E5 and E6 totally lost its antimicrobial activity. The fact that no inhibition noticed by E1, E4, E5 and E6 against indicator bacteria is an indication that antimicrobial activity were due to the production of organic acid secretion, and the reduction antimicrobial activity of E2 and E3 is an indication that there are other antimicrobial substance such as bacteriocin, diacetyl or hydrogen peroxide [14]. The clearly inhibition zone of neutralized CFS shown in **Figure 6.**

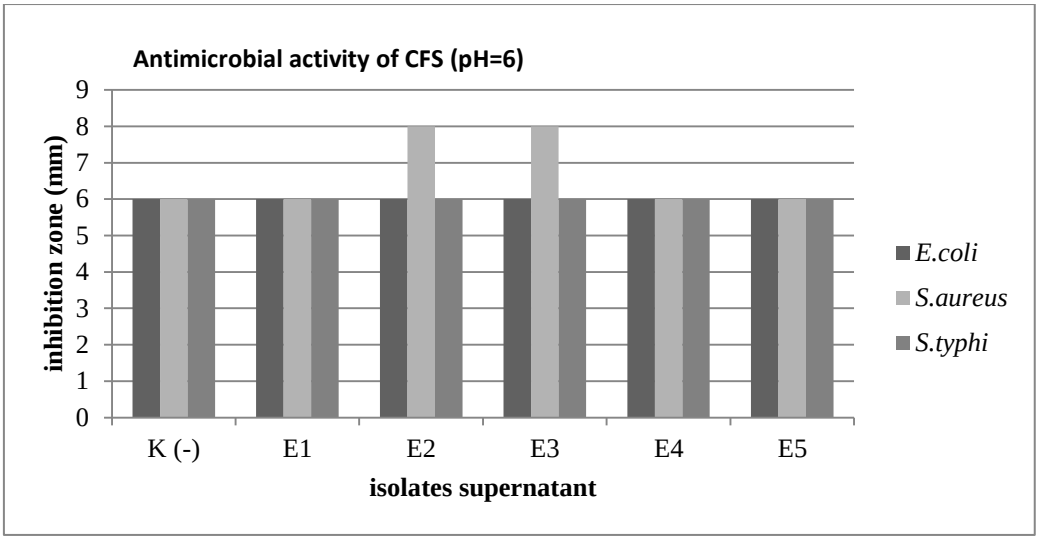
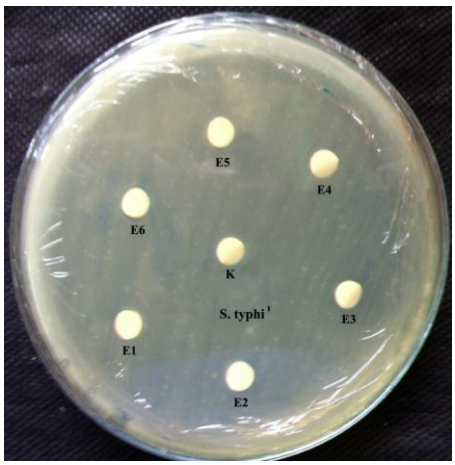
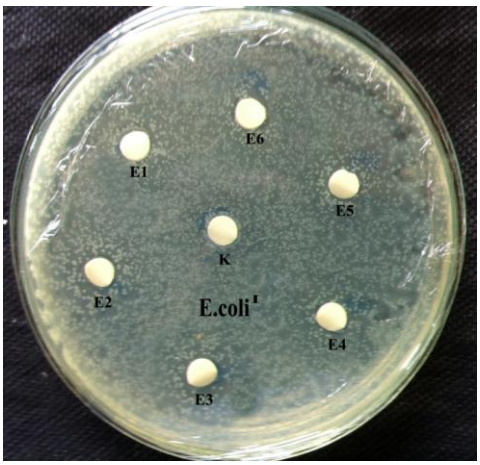


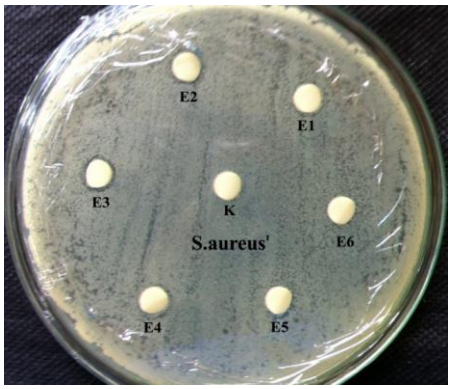
Figure 5: Antimicrobial activity of neutralized CFS against pathogen bacteria



(a)



(b)



(c)

Figure 6: Inhibition zone of neutralized CFS against pathogen bacteria : (a) against *S.typhi* (b) against *E.coli* (c) against *S.aureus*

ISSN: 0975-8585

Heat Sensitivity of CFS LAB on Pathogen Bacteria

Figure 7 shows that isolate E2 has a better antimicrobial activity than E3. The inhibition zone of E2 against *E.coli* ranging from 10.2 mm to 12mm, E3 ranging from 10mm to 11mm. E2 showed a stable activity against S.aureus with inhibition zone was 12mm and inhibition zone of E3 against *S.aureus* ranging from 10.1mm to 12mm. The stable inhibition zone against *S.aureus* indicated that there are antimicrobial substance which stable in higher temperature, for example, bacteriocin. Other studies have reported that *Bacterocin Like Substance* (BLS) produced by 8 LAB isolates which isolated from cheeses and yoghurt still have a stable antimicrobial activity after heated at 80°C and 100°C for 90minutes, but at 121°C the antimicrobial activity reduced or totally lost against *Listeria monocytogenes* [18]

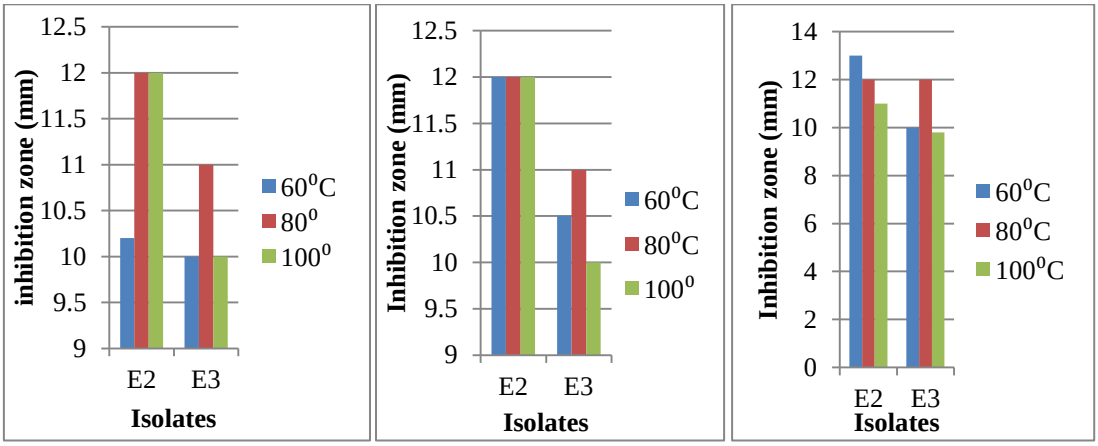


Figure 7: The antimicrobial activity of CFS LAB after exposure of various temperature against : (a) *E.coli* (b) *S.aureus* (c) *S.typhi*

Estimation of Protein Concentration using Bradford Method

Table 2 shows the extra cellular protein concentration of Lactobacillus sp using Bradford method. Based on BSA calibration curve the regression linear equation was y = 0.0282x , R = 0.9974. with x = protein content of bacteriocin and y = absorbance value crude bacteriocin with λ = 595nm. The protein concentration of crude bacteriocin determined by multiplying protein concentration in cuvette with dilution factor. The mean concentration of protein was 1.45mg/ml.

Molecular Size Determination Samples by Tris Glycine SDS PAGE

SDS PAGE result shows that there is a protein fragment below 10 kDa that it might be a bacteriocin like substance. Parada *et al* [17] classified the bacteriocin based on biochemical properties and genetic characteristic.Class I bacteriocin (<5kDa) and Class II bacteriocin (<10kDa) stable with high temperature. Molecular size of class III bacteriocin ranging from 30-300kDa and sensitive to heat. We assumed that the bacteriocin produced by isolate E2 might be class II bacteriocin. The estimation of molecular size of bacteriocin shown in **Figure 8**

Temperature stability is important if the bacteriocin will used as food preservative because in many step food processing involved a high temperature [2]. Soomro and Masud [19] have reported that *Lactobacillus acidophilus* produce bacteriocin with molecular size 6.5kDa, indicated the bacteriocin is class II bacteriocin which have stable antimicrobial activity against *E.faecalis*, *E.coli* and *S.aureus* after heating at 100°C for 30 minutes. Masayuki *et al* [20] have reported the antimicrobial activity of *Lactobacillus acidophilus* was stable after heated at 100°C for 15 minutes and the molecular size was 14.4kDa.

ISSN: 0975-8585

Table 2: Determination of protein concentration using Bradford method

Cuvette no	1	2	3	4
Aquabides(µl)	800	798	796	794
Crude bakteriocin	-	2	4	6
Bradford reagent(µl)	200	200	200	200
A_{595}nm		0.084	0.165	0.238
Protein (µg)	-	2.97	5.85	8.43
Concentration in cuvette (µg/ml)	-	2.97×10^{-3}	5.85×10^{-3}	8.43×10^{-3}
Dilution factor	-	500	250	167
Crude bacteriocin concentration(mg/ml)	-	1.48	1.46	1.40

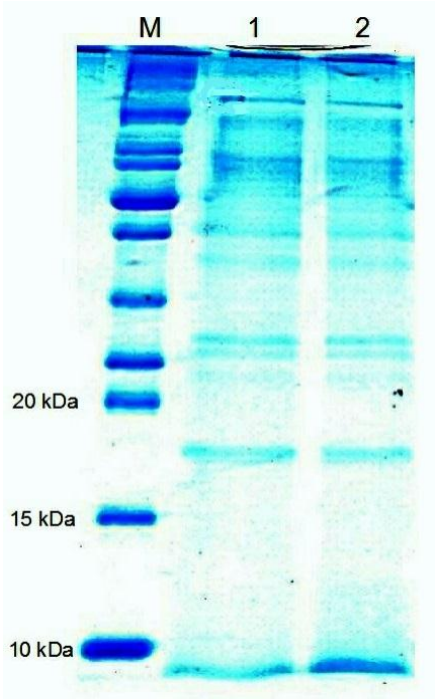


Figure 8: SDS PAGE result of E2; M: protein ladder, 1 : crude bacterocin with 60% precipitation, 2 : crude bacteriocin with 70% precipitation

CONCLUSION

Finally it may be concluded that *Lactobacillus* sp isolated from "dadih", a fermented traditional food from West Sumatera, Indonesia, produced bacteriocin like substance that has spectrum antimicrobial activity against *S.aureus*. Partially purified bacteriocin is characterized by heat stability at 60°C, 80°C, and 100°C. Molecular size of the crude bacteriocin is below 10kDa, indicated that the bacteriocin might be class II bacteriocin which heat stable bacteriocin

ACKNOWLEDGEMENT

I thank to my master student Edy Fachrial for his excellent research and DNA Molecular Group in University of Andalas, Padang, Indonesia. My great gratitude also address to the Rector and Director of Research at University of Andalas, Padang, Indonesia for supporting grand research no : 023.04.15061/2014 December 5th, 2013

ISSN: 0975-8585

REFERENCES

[1] Liasi SA Azmi, TI Hassan, MD Shuhaimi, M Rosfarizan, M Ariff, AB. Malaysian J Microbiol 2009; 5(1) : 33-37

[2] Banerjee SP, Dora KC, Chowdhury S. J Food Sci Technol 2013; 50 (1) : 17-25

[3] Agrawal N, Prakash A. Internet J Food Saf 2013; 15: 39-42

[4] Mohammed SSD, Ijah UJJ. Ann Food Sci Technol 2013 14 ; 122-128.

[5] Taheri P, Samadi N, Khoshayand M R, Fazeli M R, Jamalifar H, Ehsani M R. Int J Agr Res 2011; 2 : 27-34.

[6] Rozila A, Suryani IE, Lani MN, Sharina MD, Hasmah MS, Asma H, Sharida MD.UMT11th International Annual Symposium on Sustainability Science and Management 2011; 540-543

[7] Askari GA, Kahouadji A, Khedid K, Charof R, Mennane Z. J Sci Res 2012; 11(2) : 209-2012

[8] Rattanachaikunsopon P, Phumkachorn P. Scholars Research Library 2010; 4**:** 218-228.

[9] Lahtinen S, Ouwenhand AC, Salminen S, Wright AV. Lactic Acid Bacteria, CRC Press 2012, pp.2

[10] Arokiyamary A, Sivakumar PK. Curr Bot 2011; 2(3) : 05-08.

[11] Cleveland J, Montville TJ, Nes IF, Chikindas ML. Int J Food Microbiol 2001; 71 : 1-20

[12] Ambri K, Kusnadi J, and Putri WDR. Agr Technol J 2009 ; 10: 1-9.

[13] Iranmanesh M, Ezzatpanah H, Mojgani N, Torshizi MAK, Aminashar M, Maohamadi M. European J Food Res Rev 2012 ; 2 (3) : 79-92

[14] Mbawala A, Mahbou PY, Mouafo, HT, Tatsadjieu LN. The J Animal Plant Sci 2013; 23(1) : 157-166

[15] Caplice E, Fitzgerald GF. J Food Microbiol 1999. 50; 131- 149

[16] Saranya, S and Hemashenpagam, N. Int J Microbiol Res 2012; 5(1) : 341-348

[17] Parada JL, Caron CR, Medeiros ABP, Soccol CR. Brazilian Arch Biol Technol 2007 ; 50 : 521-542

[18] Yang E, Fan L, Jiang Y, Doucette C, Fillmore S. AMB Express 2012. 2(48) : 1-12

[19] Soomro AH, Masud T. The Australian J Dairy Technol 2008; 63(1) : 8-14

[20] Masayuki Y, Ozaki K, Ota F. Microbiol Res 2003; 158: 169-172