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Antimicrobial Activity of Lactic Acid Bacteria Isolated from *Budu* of West Sumatera to Food Biopreservatives

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Abstract: *Budu* is a traditional fermented fish product which are produced and distributed within a specific area in the province of West Sumatera prepared from Spanish mackerel *(Scomberomorus guttatus)*. The samples were obtained from the small scale domestic factory. The aim of this research are to isolate and identify Lactic Acid Bacteria (LAB) activity from *budu* with pathogen bacteria *(Escherichia coli, Staphylococcus aureus, Salmonella thypi, Bacillus subtilis* and *Listeria monocytogenes)* followed identified by PCR. The bacteria isolated were growth on medium Glucose Tripton Agar (GTA) supplement with $CaCO_3$ and then performed purification by plate out on *deMan Rogosa and Sharpe* (MRS) *Agar*. It was found 138 isolates of LAB showed with clear zone around the culture and 8 isolates (82, 84, 85, 89, 811, 813, 828 and 834) has been antimicrobial activity against the growth of pathogenic bacteria. The results showed that isolates 828 had the highest antimicrobial activity against all bacteria test, with a range of inhibition zone 14-35 mm, gram positive, spore former bacil, motility and catalase positive. Based on morphological examination and PCR analysis, the isolate 828 was primarily identified as *Bacillus cereus* strain HVR22 bacteria.

Key words: Antimicrobial activity, *Budu*, isolated, food biopreservatives, West Sumatera

INTRODUCTION

Antimicrobial agent is a general term used to refer to any compound which include antibiotics, food antimicrobial agents, sanitizer, disinfectants and other substances that acts against microorganisms (Katzung, 2004). Antimicrobial agents can be produced by fermentation products such as milk, vegetables and fish, such: *plasom* of Thailand (Kopermsub and Yunchalard, 2010) (Hwanhlem *et al*., 2011), bakasang of Indonesia (Ijong, and Ohta, 1996) and Budu of Malaysia (Liasi *et al*., 2009) and budu from West Sumatera.

Budu from West Sumatera differs from the Malaysian budu, not only in the type of fish but also in the processing. In Malaysia budu is produced using anchovies (*Stolephorus* sp.), the small sized marine fish locally known as *ikan bilis*, as the raw material. Anchovies are mixed with coarse salt at a ratio of 3:2 and fermented a large covered earthen container, concrete tank or black polyethylene tank for more than 8 months in an unshaded open area. After fermentation is complete, anchovy fries is naturally degraded into a dark brown liquid (Huda and Rosma, 2006). This fries mixed with tamarind and coconut sugar in salt solution (15%) and then fermented at room temperature in large earthen ware post (Liasi *et al*, 2009).

The *budu* from West Sumatera is a fermented fish product mainly in the coastal areas such a Pariaman, Tiku and Pasaman. *Budu* normally made from bigger sized marine fish such as Spanish mackerel (*Scomberomorus* sp.) and leatherskin (*Chorinemus* sp.), locally, known as *ikan tenggiri* and *ikan talang*. Reported that traditional processing of West Sumatera *budu* starts with hanging the fresh fish by its caudal fin at room temperature for 30 h. Once the fish is marked by a rift bulging fish meat, the fish is cut into a butterfly style and all of the intestinal organs and gills are removed. The fish is then washed to ensure that it is free of blood and other intestinal residues. The fish layered with coarse salt at a ratio of 1:5-1:3 for 3-4 h. After which the fishes rinsed to remove the excess salt. The fish is then sun-dried for 4-5 days.

Antimicrobials have been used increasingly as a primary intervention for inhibition or activation of pathogenic microorganisms in foods (Davidson and Zivnovic, 2003). Generally, in fermented foods, Lactic Acid Bacteria (LAB) display numerous antimicrobial activities. This is mainly due to the production of antimicrobial metabolites including lactic acid, hydrogen peroxide (H_2O_2), diasetil, carbon dioxide (CO_2) and bacteriocins and antifungal peptides.

Bacteriocins are the peptides and protein antibiotics which are produced by several species and have antimicrobial properties usually against other closely related species (Olivera *et al*., 2004). These

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antimicrobial peptides are gaining more and more attention not only as an alternative therapeutic agent for the prevention and treatment of infections but also as preservatives in food industries to avoid deterioration and spoilage of food (Anthony *et al*., 2009). Bacteriocins are generally recognized as naturally occurring food preservatives able to influence the quality and safety of foods (Settanni and Corsetti, 2008). The aims of this research are to isolate and identify lactic acid bacteria from traditional fermented fish product "*budu*" and to screen and determine the activity antimicrobial with pathogen bacteria (*Escherichia coli*, *Staphylococcus aureus*, *Salmonella thypi*, *Bacillus subtilis* and *Listeria monocytogenes*) in order to apply for food biopreservatives.

MATERIALS AND METHODS

Three samples of budu were collected in sterile plastics from manufactures small scale domestic factory in West Sumatera (Sirah river and Gasan district Padang Pariaman and Sasak district Pasaman). The samples were transferred immediately to the laboratory for microbiological analysis.

Pathogenic bacteria strains and culture medium: *Escherichia coli*, *Staphylococcus aureus*, *Salmonella thypi*, *Bacillus subtilis* and *Listeria monocytogenes*. The culture medium in this research: GTA+$CaCO_3$ plate a consisted of 20 gr glucose, 2, 5 gr tripton, 20 gr bacto agar (Oxoid) and 15 gr $CaCO_3$, MRS broth (Merck) and Nutrient Agar (NA) (Merck).

Isolation of lactic acid bacteria: At first, the samples were homogenized in mortar and pestle, taking 10 g of well mixed sample blended in 90 ml of 0, 85% (w/v NaCl) physiological saline. Further serial dilutions to 10^{-8}. For isolation of LAB, of each dilute 0, 1 ml inocula were plated out GTA+$CaCO_3$ and the plates were incubated at room temperature for 48-72 h. The existence of clear zone around the colonies suspected to be the lactic acid bacteria. Colonies were selected at random from plates and the isolates purified by repeated streaking onto MRS agar. Purified strains of LAB were inoculated into MRS broth (pH 6.5) and incubated 24 h at 30°C. All purified strains were kept in MRS agar containing 20% glycerol at -20°C (Modified Rahayu and Margino, 1997).

Characterization of lactic acid bacteria: The identified of the isolates were determined by the standard procedure of gram staining, catalase test, motility and spore former test (Hadioetomo, 1985; Fardiaz, 1989; Lay, 1994). Characterization bacterial strains of LAB determination the using standard methods "Manual for the identification of medical bacteria" (Cowan and Steel's, 1975).

Antimicrobial activity test: The modified methods of Savodogo *et al* (2004) and Girum *et al* (2005) were used to determine the antibacterial activities of the isolates. A single isolated colonies were selected from MRS agar plates and transferred to grow in sterile MRS broth. The broth culture was incubated erobically at 37°C for 48 h. After incubation, the culture was centrifuged at 10.000 rpm for 20 minute at 4°C to obtain the culture supernatant. The indicator microorganisms (*Escherichia coli*, *Staphylococcus aureus*, *Salmonella thypi*, *Bacillus subtilis* and *Listeria monocytogenes*) were grown in NA for 24 h at 37°C. A sterile cotton swab was dipped into the culture of the indicator microorganisms and rotated several times and the swab was then pressed firmly on the inside wall of the tube above the fluid level to remove excess inoculums. The dried surface of NA was inoculated by streaking the swab over the entire agar surface. This procedure was repeated by streaking two or more times while rotating the plate each time to ensure an even distribution of inoculums. For the bioassay, the sterile filter disc was dipped into the culture supernatant and touched to the side of container to remove excess liquid and it was then placed on a NA plate. After 24 hours at 37°C of aerobic incubation, each plate was evaluated and the diameters of the inhibition zones, including the diameter of the disc, were measured using a transparent ruler (Assefa *et al*, 2008).

DNA extract, PCR and sequencing: The Genomic DNA was extracted from pure culture of the *Bacillus* isolate using genomic DNA extraction kit following instructions of the Pitcher *et al* (1989, modified) (White *et al*., 1990). The 1.5 kb 16S rDNA gene were amplified by PCR using a pair of universal bacterial 16S rDNA gene primers 9F: 5-AAG GAG GTG ATC CAG CC-3 and primers1541 R: 5-GAG TTI GAT CCT GGC TCA G-3. The PCR was carried out according to (Hiraishi *et al*., 1995). To sequence part of the 16S-rRNA genes, the 16S-rRNA genes were amplified by PCR using specific primers of 63f and 1387r from genomic DNA (200 ng) on Ready-To-GO PCR Beads (Pharmacia-Biotech, Uppsala, Sweden). Phenol-chloroform-isoamyl alcohol (25:24:1) treatment, ethanol precipitation and agarose gel electrophoresis were used to purify the genomic DNA. Total volume of the PCR reaction (25 ul) consisted of 1.5 U *Tag* DNA Polymerase, 10mM Tris-HCl (pH 9 at room temperature), 50 mM KC1, 1.5 mM MgCb, 200 uM of each dNTPs and stabilizer including BSA. The reaction was incubated in a Gene Amp PCR System 2400 Thermocycler (Perkin-Elmer Cetus, Norwalk, Conn.). Part of 16S-rRNA gene was sequenced to infer the closest related organism from the Ribosomal Database Project (RDP) maintained at the University of Illinois, Urbana-Champaign. The sequencing reactions were done by using the Big Dye Ready Reaction Dye Deoxy Terminator kit, purified by

ethanol-sodium acetate precipitation. The reactions were run on an ABI PRISM 3130 Genetic Analyzer (PE Applied Biosystems, Foster City, CA).

RESULTS AND DISCUSSION

Isolation of LAB: The isolation of LAB from budu was performed GTA supplemented with $CaCO_3$ which was obtained at the room temperature, 72 h of incubation and MRS agar was used as a preliminary screening medium for LAB. It was found that 138 isolates exhibited a clear zone and growth on MRS agar. Calcium carbonate was used as an indicator for acid-producing strains since it dissolved when interact with acid then a clear zone is observed. All of isolates were identified as LAB using the criteria of being gram positive and catalase positive. Of these, 135 isolates (98%) from budu were identified as rod shape and 3 (2%) isolates were identified as cocci shape.

In the production of fermented foods, the initial food matrix presents abiotic and biotic conditions that select for the growth of specific microbial communities (Giraffa, 2004). In the case of *budu*, the initial concentration of salt select for the growth of lactic acid bacteria. Hwanhlem *et al.* (2011) reported of lactic acid bacteria from Thailand fermented fish *(plasom)* were identified 133 isolates, 25 isolates were cocci, 75 isolates were short rods and 33 isolates were rods. Tanasupawat *et al.* (2009) observed from fish sauce *(nam-pla)* in Thailand have fifteen isolates were moderately rod-shaped halophilic extremely, rod-shaped halophilic bacteria and sphere shaped bacterium. Kim *et al.* (2002) isolated *Bacillus* sp. SFF34, from Korean traditional fermented flatfish. Anihouvi *et al.* (2006) from *Ianhouin*, a fermented fish product in Republic Benin get *Micrococci* and *Bacilli* were the predominant organisms enumerated in product.

Detection of antagonistic activity: A total 138 isolated randomly picked from *budu* samples for the morphological identification was selected isolated gave 8 (B2, 84, 85, 89, 811, 813, 828 and 834) isolates has the most extensive zone of inhibition when compared to other isolates. Based on the data it appears that the fluid extracellular of eight isolates of the inhibition of the bacterial pathogen/indicator and it appears that the inhibitory activity extracellular 84 and 828 had a higher power resistor. While isolates had the highest inhibitory power is 828 isolates with inhibition of the *Escherichia coli* 14 mm, against *Staphylococcus aureus* 18 mm, against *Salmonella thypi* 15 mm, against *Bacillus subtilis* 35 mm and the *Listeria monocytogenes* 19 mm (Table 1).

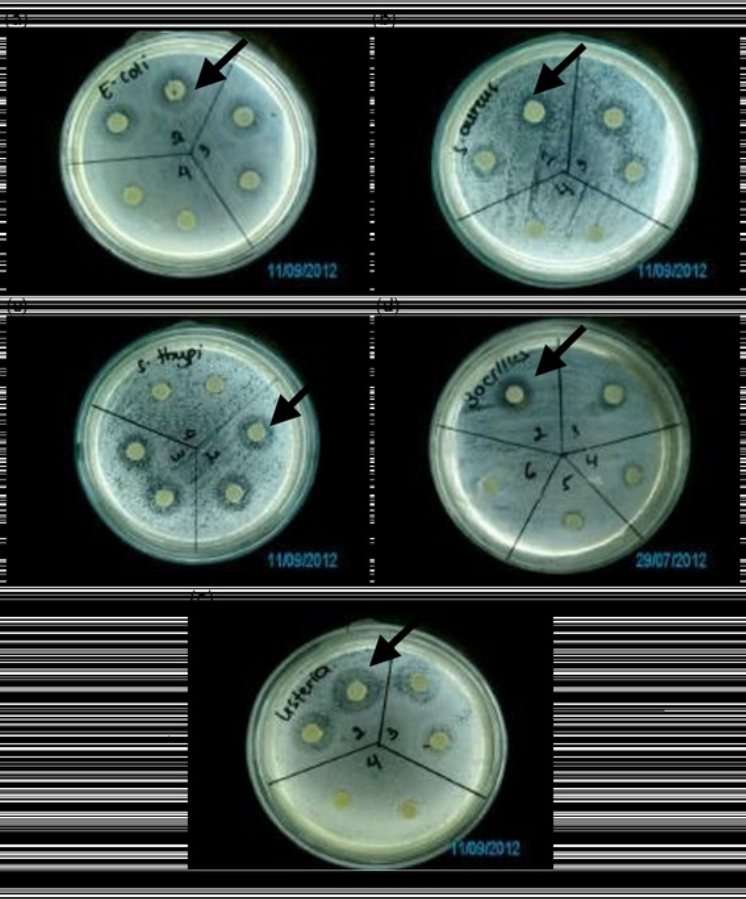
Isolates that have the widest zone of inhibition against *Escherichia coli* isolates 84 which is 20 mm with inhibitory activity mm^2/ml 14287, against *Staphylococcus aureus* isolates 84 is the inhibition zone diameter of 24 mm with inhibition activity 21217 rnmvml, the bacteria *Salmonella thypi* is isolate 82, 85, 811 and 828 that is measuring 15 mm by 7418.25 mm^2/ml inhibition activity, the bacterium *Bacillus subtilis* is isolat 813 and 828 that is inhibition zone diameter 35 mm with inhibition activity 46668.250 mrnvrnl and the bacteria *Listeria monocytogenes* is isolate 82, 85, 89, 811 and 834 that is 20 mm wide with 14287 mrrrrml inhibition activity. The results showed an average inhibition zone of *Escherichia coli* ranging from 10 mm (isolate 834) to 20 mm (isolate 84), *Staphylococcus aureus* ranging from 13 mm (isolate 813 and 834) to 24 mm (isolate 84), *Salmonella thypi* ranging from 10 mm (isolate 834) to 15 mm (isolate 82, 85, 811 and 828), *Bacillus subtilis* ranging from 19 mm (isolate 85) to 35 mm (isolate 813 and 828) and *Listeria monocytogenes* ranging from 15 mm for isolate 813 to 20 mm for isolate 82, 85, 89, 811 and 834.

Table 1: Antibacterial activity of 8 isolates of LAB obtained from *budu* samples against different indicator microorganisms

No. of strains	Diameter of inhibiton zone (mm)				
	E coli	*S. aureus*	*S. thypi*	*B. subfilis*	*L. monocyfogenes*
82	13	20	15	20	20
84	20	24	13	20	18
85	12	21	15	19	20
89	12	16	11	20	20
811	12	16	15	23	20
813	12	13	12	35	15
828	14	18	15	35	19
834	10	13	10	33	20

According to Ammor *et al.* (2006) LAB produce a wide range of products from low molecular mass compounds, such as hydrogen peroxide, carbon dioxide and diacetyl, to high molecular mass compounds, such as bacteriocins. Organic acid produced by LAB leads to a reduction in pH levels and increases the production of hydrogen peroxide (Ponce *et al.*, 2008), enzymes (lactoperoxidase system with hydrogen peroxide and lysozyme), low-molecular metabolites (reuter in, diacetyl and fatty acids) and bacteriocins (nisin and others) (Holzapfel *et al.*, 1995). These products exhibit antibacterial activity against various pathogenic microorganisms, including gram-positive and gram-negative bacteria (Maragkoudakis *et al.*, 2009).

Savodogo *et al.* (2004) isolated eighty strains of lactic acid bacteria producing bacteriocin were isolated from Burkina Faso fermented milk samples. These strains were identified to species *Lactobacillus fermentum*, *Pediococcus* sp., *Leuconostoc mesenteroides* and *Lactococcus*. Isolated bacteriocin exhibited antibacterial activity against *Enterococcus faecalis Bacillus cereus Staphylococcus aureus* and *Escherichia coli* using the agar drop diffusion test. The inhibition diameters obtained with bacteriocin are between 8 and 12 mm. Gram positive indicator bacteria were most inhibited. Ogunshe *et al.* (2007) isolated 50 bacteriocin producing


(a)
E-coli
11/09/2012
(b)
S. aureus
11/09/2012
(c)
S. thygi
11/09/2012
(d)
Bacillus
29/07/2012
(e)
Listeria
11/09/2012

GGCGGCGTGCCTAATACATGCAAGTCGAGCGAATGGATTAAGAGCTTGCTCTTATGAAGT
TAGCGGCGGACGGGTGAGTAACACGTGGGTAACCTGCCCATAAGACTGGGATAACTCCGG
GAAACCGGGGCTAATACCGGATAACATTTTGAACCGCATGGTTCGAAATTGAAAGGCGGC
TTCGGCTGTCACTTATGGATGGACCCGCGTCGCATTAGCTAGTTGGTGAGGTAACGGCTC
ACCAAGGCAACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACTGAGACA
CGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGACGAAAGTCTGA
CGGAGCAACGCCGCGTGAGTGATGAAGGCTTTCGGGTCGTAAAACTCTGTTGTTAGGGAA
GAACAAGTGCTAGTTGAATAAGCTGGCACCTTGACGGTACCTAACCAGAAAGCCACGGCT
AACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTATCCGGAATTATTGGG
CGTAAAGCGCGCGCAGGTGGTTTCTTAAGTCTGATGTGAAAGCCCACGGCTCAACCGTGG
AGGGTCATTGGAAACTGGGAGACTTGAGTGCAGAAGAGGAAAGTGGAATTCCATGTGTAG
CGGTGAAATGCGTAGAGATATGGAGGAACACCAGTGGCGAAGGCGACTTTCTGGTCTGTA
ACTGACACTGAGGCGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCAC
GCCGTAAACGATGAGTGCTAAGTGTTAGAGGGTTTCCGCCCTTTAGTGCTGAAGTTAACG
CATTAAGCACTCCGCCTGGGGAGTACGGCCGCAAGGCTGAAACTCAAAGGAATTGACGGG
GGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAG
GTCTTGACATCCTCTGAAAACCCTAGAGATAGGGCTTCTCCTTCGGGAGCAGAGTGACAG
GTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGC
GCAACCCTTGATCTTAGTTGCCATCATTAAGTTGGGCACTCTAAGGTGACTGCCGGTGAC
AAACCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCTTATGACCTGGGCTACAC
ACGTGCTACAATGGACGGTACAAAGAGCTGCAAGACCGCGAGGTGGAGCTAATCTCATAA
AACCGTTCTCAGTTCGGATTGTAGGCTGCAACTCGCCTACATGAAGCTGGAATCGCTAGT
AATCGCGGATCAGCATGCCGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCA
CACCACGAGAGTTTGTAACACCCGAAGTCGGTGGGGTAACC

Fig. 2: Nucleotide sequence of *Bacillus* sp 828 by 16S rDNA sequence (full sequence; 1.462 base pairs)

Belonging to the genus *Bacillus* belong to class I of the phylum Firmicutes i.e., the bacilli. Members of the genus *Bacillus* are gram-positive, aerobic and endospore-forming bacteria that are characterized by their rod-shaped cell morphology, catalase production and their ubiquitous distribution. They are found in diverse environments such as soil and clays, rocks, dust, aquatic environments, vegetation, food and the gastrointestinal tracts of various insects and animals (Nicholson, 2002).

Members of the *Bacillus* group *sensu-lato* are considered good producers of antimicrobial substances, including peptide and lipopeptide antibiotics and bacteriocins (Stein, 2005). The production of antimicrobial substances and sporulation capacity confer *Bacillus* strains with a double advantage in terms of their survival in different habitats. The presence of *Bacillus* species in food does not always imply spoilage or food poisoning and some species or strains are even used in human and animal food production such as, for example, *Bacillus subtilis* strains that are used in Natto, an East Asian fermented food production (Hosoi and Kiuchi, 2003). Furthermore, specific *B. subtilis* strains are also used as a starter culture for fermenting soybeans into the traditional West African condiment dawadawa (Terlabie *et al*, 2006) or for fermenting African mesquite seeds in the production of the Nigerian food condiment okpehe (Oguntoyinbo *et al*, 2007). A nontoxinogenic *Bacillus cereus* ssp. toyoi with probiotic properties is also used as an animal feed additive (Lodemann *et al*, 2008).

Bacillus is an interesting genus to be investigated for antimicrobial activity because *Bacillus* sp. produces a large number of peptides with biological activities. e.g., cerecin 7 (Oscariz *et al*, 1999) produced by *B. cereus* BC7, tochicin (Paik *et al*, 1997) from *B. thuringensis*, thuricin 7 (Cherif *et al*, 2003), subpeptin JM4-A and subpeptin JM4-B produced by *B. subtilis* JM4 (Wu *et al*, 2005). The chemical and physical diversity of peptide antibiotics makes them ideal candidates not only for therapeutic applications but also in other areas, especially the agri-food industry.

Identification of *lacticacid bacteri* by 16SrRNA: Methods for the detection and identification of *B. cereus* are e.g., serotyping, pyrolytic gas chromatography, pyrolytic mass spectrometry, ribotyping, phage typing, plasmid profiles, electrophoresis in pulse electric field and Polymerase Chain Reaction (PCR) using genera-specific and species-specific primers. Isolate 828 was DNA extraction for GES methods (Pitcher *et al*, 1989; Modified), amplification PCR of its 16S rDNA with specific primers 9F: 5'-AAG GAG GTG ATC CAG CC-3' and primers 1541 R: 5'-GAG TTT GAT CGT GGC TCA G-3' (White *et al*, 1990), purification PCR product by PEG precipitation methods (Hiraishi *et al*, 1995) and ethanol purification methods. Result of purification to analysis against by automated DNA sequencer (ABI PRISM 3130 Genetic Analyzer, Applied Biosystems), thus for trimming and assembling by BioEdit program at BLAST program at the NCBI database, showed that the new isolate was taxonomically very close to *Bacillus cereus* strain HVR22 (Fig. 1).

Bacillus cereus produces several Bacteriocin like Inhibitory Substances (BLIS) or cereins, such as cerein 8A produced by *B. cereus* BA (Bizani and Brandelli, 2002; Bizani *et al*, 2005), cerein GN105 produced by *B. cereus* GN105 (Naclerio *et al*., 1993) and cereins produced by *B. cereus* strains 30/11, 15/5, 8/10 and 8/2 (Torkar and Matijas, 2003). Cerein 8A from *B. cereus* BA isolated from soil of native woodlands of Southern Brazil (Bizani and Brandelli, 2002) is protease sensitive, stable in the pH range of 2-11 and relatively thermostable, only losing activity at temperatures 47°C for 30 min (Bizani *et al*, 2005). This cerein has an apparent molecular weight of 26 kDa by SOS-PAGE and contains acyl group (s) and aromatic amino acids such as phenylalanine and tyrosine residues which may play a critical role in antimicrobial activity (Sheehan, 2002; Bizani *et al*., 2005). Cerein 8A inhibits several pathogenic and food-spoilage microorganisms such as *L. monocytogenes* and *B. cereus*, apparently by disturbing their membrane function (Bizani and Brandelli, 2002; Bizani *et al*., 2005). This BLIS is also active against intact spores of *B. cereus* (Bizani *et al*, 2005).

Conclusions: The isolation of LAB from *budu* was found that 138 isolates exhibited a clear zone, 135 isolates (98%) identified as rod shape and 3 (2%) isolates as cocci shape. For the morphological identification was selected isolated gave 8 (B2, B4, B5, B9, 811, B13, B28 and 834) isolates has been antimicrobial activity against the growth of pathogenic bacteria (*E. coli*, *B. cereus*, S. *thypi*, *B. subtilis* and L. *monocytogenes*). Isolates B28 had the highest antimicrobial activity against all bacteria test, with a range of inhibition zone 14-35 mm, gram positive, rod shape, spore former bacil, motility and catalase positive, Based on morphological examination and PCR analysis, the isolate 828 was primarily identified as *Bacillus cereus* HVR22 strain bacteria.

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