

Characterization of Antimicrobial Bacteriocin Produced by *Bacillus cereus* SS28 Isolates from Budu, a Traditionally Fermented Fish Product of West Sumatera

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Bacillus cereus SS28 isolated from budu, a fermented fish product from West Sumatera, produced antimicrobial compound that had broad spectrum of inhibition against five microorganisms (*Escherichia coli*, *Staphylococcus aureus*, *Salmonella thypi*, *Bacillus subtilis*, and *Listeria monocytogenes*). The aims of this research are characterization of *Bacillus cereus* SS28 antimicrobial activity and observation of its effect to the cellular morphology of *Staphylococcus aureus* with electron microscope. Antimicrobial compound produced by *Bacillus cereus* SS28 was stable at pH range between 2 and 11 and to heating at 121 °C for 15 min. Maximum antimicrobial activity was expressed at pH 2-3 and 70 °C for 45 min. The activity remained after 15 min exposure to UV light. The main changes observed under SEM and TEM were the alteration of *Staphylococcus aureus* structural cell membrane 48 h after exposure to the antimicrobial compound from *Bacillus cereus* SS28.

Key words: antimicrobial bacteriocin, *Bacillus cereus* SS28, budu, characterization, West Sumatera

Bacillus cereus SS28 diisolasi dari ikan budu, produk fermentasi ikan yang berasal dari Sumatera Barat yang dapat menghasilkan komponen antimikroba bakteriosin dengan spektrum yang luas dan dapat menghambat lima bakteri (*Escherichia coli*, *Staphylococcus aureus*, *Salmonella thypi*, *Bacillus subtilis* dan *Listeria monocytogenes*). Tujuan dari penelitian ini adalah melakukan karakterisasi komponen antimikroba dari bakteri *Bacillus cereus* SS28 dan mempelajari pengaruhnya terhadap morfologi sel dari bakteri *Staphylococcus aureus*. Komponen antimikroba yang dihasilkan oleh bakteri *Bacillus cereus* SS28 stabil pada perlakuan pH 2-11 dan pemanasan suhu 121 °C selama 15 menit. Aktivitas antimikroba yang paling tinggi terdapat pada pH 2-3, suhu 70 °C selama 45 menit. Bakteriosin masih stabil setelah terpapar di bawah sinar UV selama 15 menit. Dengan menggunakan SEM dan TEM terlihat perubahan struktur membran sel bakteri *Staphylococcus aureus* setelah terpapar selama 48 jam oleh komponen antimikroba dari *Bacillus cereus* SS28.

Kata kunci: antimikroba bakteriosin, *Bacillus cereus* SS28, budu, karakterisasi, Sumatera Barat

Currently the use of harmful chemicals as a preservative of food like tofu, noodles, meatballs, chicken meat, and fish is prohibited. We must search an alternatives to the particular safe food and fish preservatives and for consumption. Bacteriocins from lactic acid bacteria (LAB), especially their antibacterial activities, have attracted much attention and have been the subject of intensive investigation (Mataragas *et al.* 2002). The limited existence of data regarding bacteriocins from *Bacillus* spp. makes this genus an interesting object to investigate, since it produces diverse array of antimicrobial peptides representing several different basic chemical structures (Adetunji and Olaoye 2011).

The production of bacteriocins or bacteriocin-like substances has already been described for some *Bacillus* spp., such as *Bacillus substilis*, *B. cereus*, *B.*

stearothemophilus and other *Bacillus* spp. (Zheng 1999; Cherif *et al.* 2001; Stein *et al.* 2002). Some strains produce bacteriocin with broad spectrum of activity including important pathogens such as *Listeria monocytogenes* and *Streptococcus pyogenes* (Cherif *et al.* 2001). Some produced well characterized bacteriocins, such as lichenin and megacin produced by *B. megaterium*. Bacteriocin had also been isolated from *B. amyloliquefaciens* (Lisboa *et al.* 2006).

A number of general physicochemical properties has been studied to provide information about the composition and structure of bacteriocins. Various studies stated that bacteriocins produced by *Bacillus* sp. showed resistance to heat treatment and tolerance to pH, as described by Sharma *et al.* (2009), and Khalil *et al.* (2009) about the effects of pH, heating and exposure to UV light towards *Bacillus* sp MTCC 43 bacteriocins.

Bacillus cereus SS28 isolated from budu showed very high antimicrobial activity against all tested

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strains (*Escherichia coli*, *Staphylococcus aureus*, *Salmonella thypi*, *Bacillus subtilis* and *Listeria monocytogenes*), with range of inhibition zone 14-35 mm (Yusra *et al.* 2013). *Budu* is a fermented fish product from West Sumatera, mainly originated from the coastal areas, such a Pariaman, Tiku and Pasaman. *Budu* normally made from bigger size marine fish such as Spanish mackerel (*Scomberomorus* sp.) and leatherskin (*Chorinemus* sp.), locally, knowns as ikan tenggiri and ikan talang (Yusra 2012). However, studies related to the antibacterial characteristics of these organisms have been limited and not fully exploited. Therefore, the purpose of this research were to characterize the antimicrobial compounds isolated from *Bacillus cereus* SS28 and to observe its effect to *Staphylococcus aureus* cell morphology with electron microscopy (SEM and TEM).

MATERIALS AND METHODS

Bacterial strains. Materials used in this study were isolated from *Bacillus cereus* SS28. The indicator strains used in this work were provided by the Laboratory of Clinical Microbiology Research, Faculty of Medicine and Microbiology, Universitas Indonesia, and Laboratory Microbiology, Department of Food Science and Technology, Faculty of Agricultural Technology, Institut Pertanian Bogor. They include both gram negative and gram positive strains (*E. coli*, *S. aureus*, *S.thypi*, *B. subtilis*, and *L. monocytogenes*).

Bacterial cultures. The strain *B.cereus* SS28 provided by Yusra *et al.* (2013) was maintained at -4 °C and as frozen stock cultures in equal volumes of 10% glycerol. *B. cereus* SS28 was grown in MRS broth, *E. coli*, *S. aureus*, *S. thypi*, *B. subtilis* and *L. monocytogenes* were grown in nutrient broth (NB). The cultures were grown at 37 °C for 24 h in MRS broth or NB medium.

Growth and production of bacteriocin by *B. cereus* SS28 in a MRSB at 37 °C. *B. cereus* SS28 was grown in the MRS broth media as much as 200 ml, incubated at 37 °C for 30 h. One mL culture sample was taken hourly and put in a test tube. Changes in the optical density of the cultures were recorded at 600 nm wavelength (Olivera *et al.* 2004).

Production of Crude Bacteriocin. *Bacillus cereus* SS28 was cultivated in 250 ml erlemeyer flask containing 100 mL of MRS broth and incubated for 48 h at 37 °C. Supernatants were harvested by centrifugation

at 6000 g for 10 min at 4 °C. The pH of the cell free supernatant was adjusted to 6.5 using 1 M NaOH solution to prevent the inhibitory effect of organic acids. The supernatants were then filtered using 0.22 µm membrane filter (Millipore). The filtrates were used for the characterization of bacteriocin.

Antimicrobial Activity of Extracted Bacteriocin. Agar well diffusion and paper disc methods were used to study antimicrobial activity of the extracted bacteriocin. In the agar well diffusion assay 0.1 mL culture of the tested microorganisms (*E. coli*, *Staphylococcus aureus*, *Salmonella thypi*, *B. subtilis*, and *Listeria monocytogenes*) were spread on sterile nutrient agar. Twenty µL extracted bacteriocin preparation (CBP) was placed in each well and the plates were aerobically incubated at 37 °C for 24 hrs.

Characterization of Bacteriocin

Effect of pH on antimicrobial activity. Supernatant from *B. cereus* SS28 culture was diluted with deionized water. The diluted supernatant was then divided into several parts, each of which was adjusted to different pH levels between 2 to 11 using sterile 10 mM/l NaOH or 10 mM/l HCl solution. The solutions were then heated at 100 °C for 30 min, before the pH was adjusted to 6.5 with sterile dH₂O and assayed for its activity (Nofisulastri *et al.* 2006). The antimicrobial activity were determined by paper disc assay.

Effect of Temperature on Antimicrobial Activity. Supernatant of *B. cereus* SS28 was exposed to various heat treatments: 40, 55, 70, 85, 100, and 121 °C. Aliquot volumes of each fraction were then removed after 0, 30, 60, or 90 min and assayed for bacteriocin (Ogunbarwo *et al.* 2003).

Effect of UV Light on Antimicrobial Activity. Ten ml supernatant of *B. cereus* SS28 was placed in a sterile petri dish and exposed to short - wave UV light (wavelength 340 nm, 220-240 V, 50 Hz) situated at a distance of 30 cm from petri dishes. Time of exposure to UV light is 30 minutes after which the bacteriocin activity was estimated by the papper disc method (Ogunbarwo *et al.* 2003).

Scanning Electron Microscopy. *S. aureus* culture that has been exposed to bacteriocin from *B. cereus* SS28 at 37 °C for 48 h were examined by SEM to visualize any morphological change occurring in the cell following exposure to bacteriocins and pressure. The cell suspensions were fixed with 3% gluteraldehyde in Na-cacodylate buffer (100 mM, pH 7.1). Then the cells were pelleted and washed to

remove glutaraldehyde before resuspended in the same buffer. A drop of each suspension was transferred to a poly-L-lysine-treated silicon wafer chips that were kept for 30 min in a hydrated chamber to let the cells adhere. The attached cells were post fixed by immersing the chips in 1% osmium tetroxide (OsO_4) in cacodylate buffer for 30 min, then rinsed in the same buffer and dehydrated in ethanol in ascending concentrations (%): 50, 70, 95 (2x) and 100 (2x), for 10 min each. The chips were mounted on aluminum stubs and coated with gold-palladium in a sputter coater (Emitech K550, Ashford, Kent, England). The chips were viewed at 3 kV accelerating voltage in a Hitachi S-4000 field emission scanning electron microscope (JEM-JEOL JSM-5310LV type) and secondary electron image of cells for topography contrast were collected at several magnifications (Bolshakova *et al.* 2004).

Transmission electron microscopy. The *S. aureus* cell suspensions that has been exposed to bacteriocin from *B. cereus* SS28 at 37 °C for 48 h were harvested by centrifugation and washed twice with 0.1 M phosphate buffer (pH 7.3). The cells were fixed with 2.5% (v/v) glutaraldehyde, 2.0% (v/v) formaldehyde in 0.12 M phosphate buffer for 10 days and then postfixed in 2% (w/v) osmium tetroxide in the same buffer for 45 min. The samples were dehydrated in a graded acetone series (30-100%) and embedded in Araldite-Durcupan for 72 h at 60 °C. Thin sections

(microtome UPC-20, Leica) were mounted on grids, covered with collodion film and poststained with 2% uranyl acetate in Reynold's lead citrate. Its preparation were observed with transmission electron microscope tipe JEOL-1010 (Bozzola and Russel 1999, with modification).

RESULTS

Growth of *B. cereus* SS28 and the production of bacteriocin in MRSB at 37 °C. The growth and bacteriocin production of *B. cereus* Ss28 is slight increase of cell dry weight was observed for 28 h of fermentation. During log phase (6 - 22 hours fermentation), medium pH decreased rapidly. It occurred concurrently with the increase of the cell dry weight. The data indicated that the alteration of the medium pH was inversely proportional with growth of *B. cereus* Ss28.

Effect of pH on Antimicrobial Activity. The effect of pH on bacteriocin activity was studied. It was observed that bacteriocin produced by *B. cereus* SS28 was stable between pH 2-11 (Fig 2).

Effect of Temperature on Antimicrobial Activity. The inhibitory activity towards the test isolates was heat stable (Fig 3). The antimicrobial activity remained constant after heating at 121 °C for 15 minutes. The activity was highest when being heated at 70 °C for 45 min.

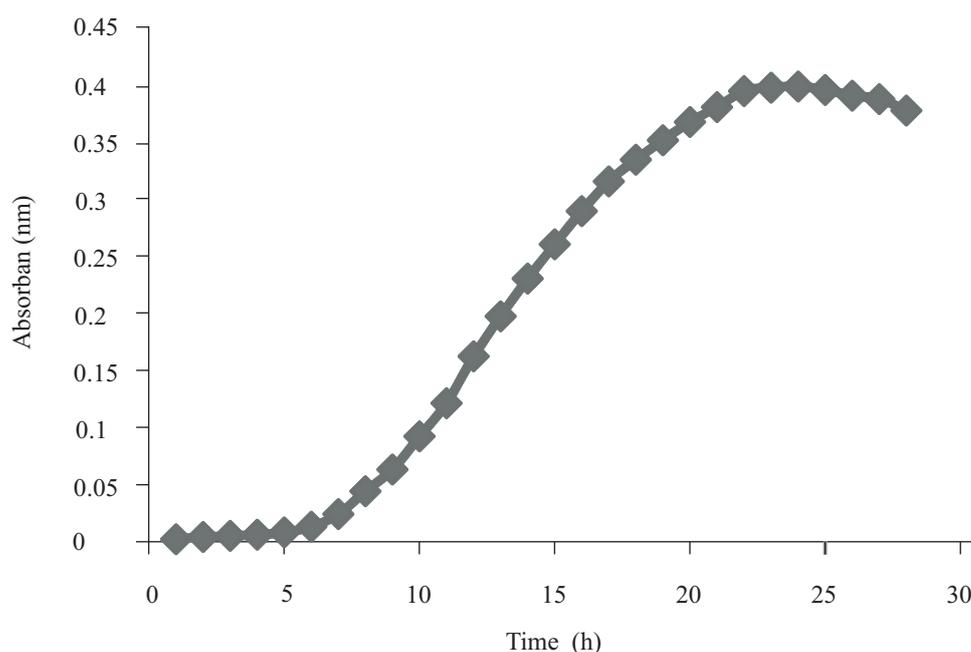


Fig 1 The growth curve of *Bacillus cereus* SS28 isolate on MRS broth medium

Effect of UV Light on antimicrobial activity. Bacteriocin produced by the test isolates was tested for their sensitivity (loss of activity) to UV light exposure. The antimicrobial activity was lost or unstable after exposure to UV-light for 15 and 30 min (Fig4).

Scanning Electron Microscopy. SEM has been

widely used in microbiology to study the surface structure of biomaterials and to measure cell attachment and changes in morphology of bacteria. The SEM-generated photomicrograph of pathogen *S. aureus* after treatment with antimicrobial compound from *B. cereus* SS28 is presented in Figure 5.

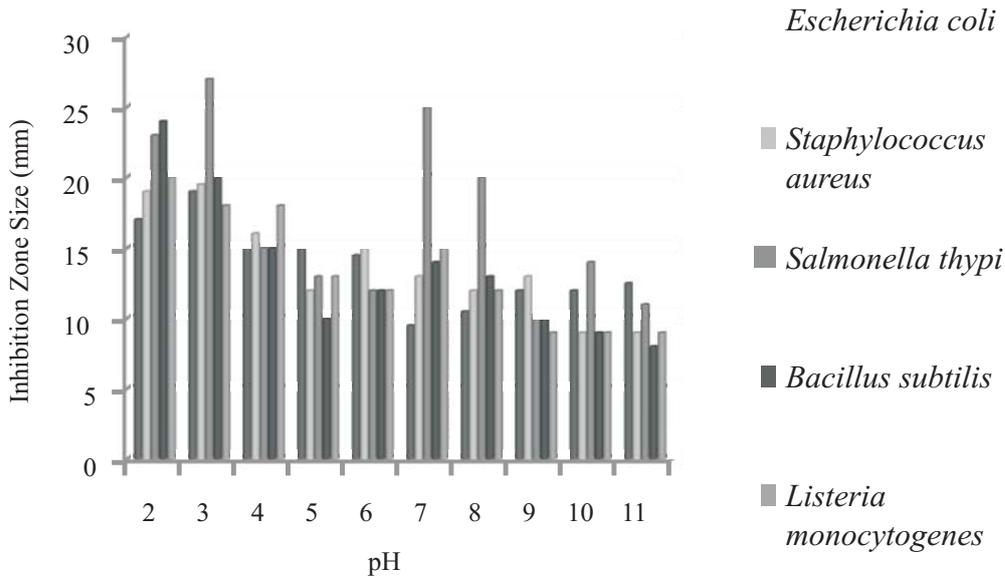


Fig 2 Effect of pH on the activity of antimicrobial compound from *Bacillus cereus* SS28 determined based on the size of the inhibition zone (mm).

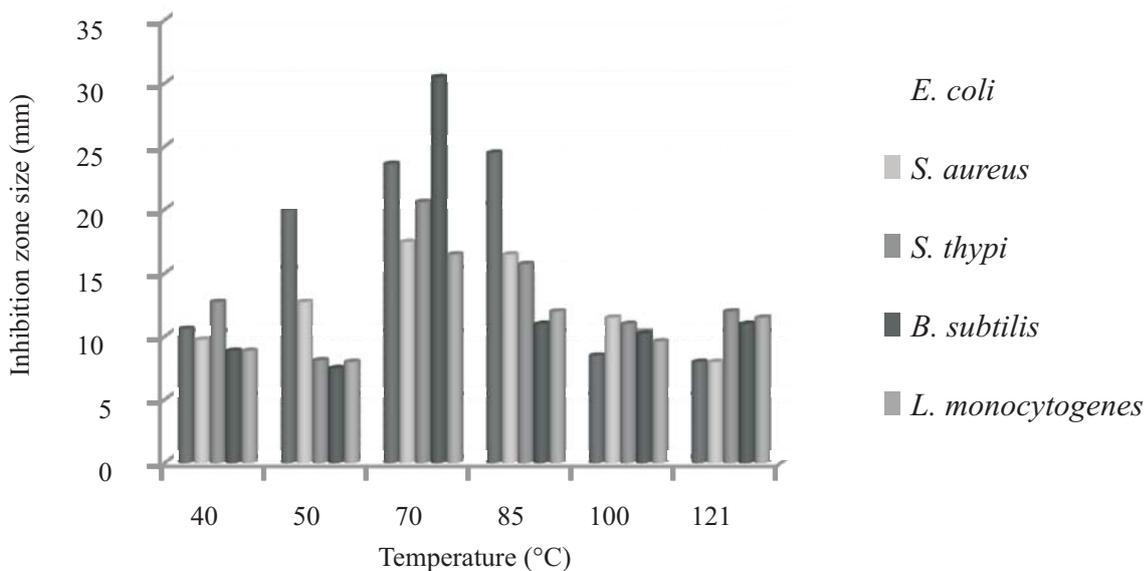


Fig 3 Effect of temperature on activity of antimicrobial compound from *Bacillus cereus* SS28, determined based on the size of the inhibition zone (mm).

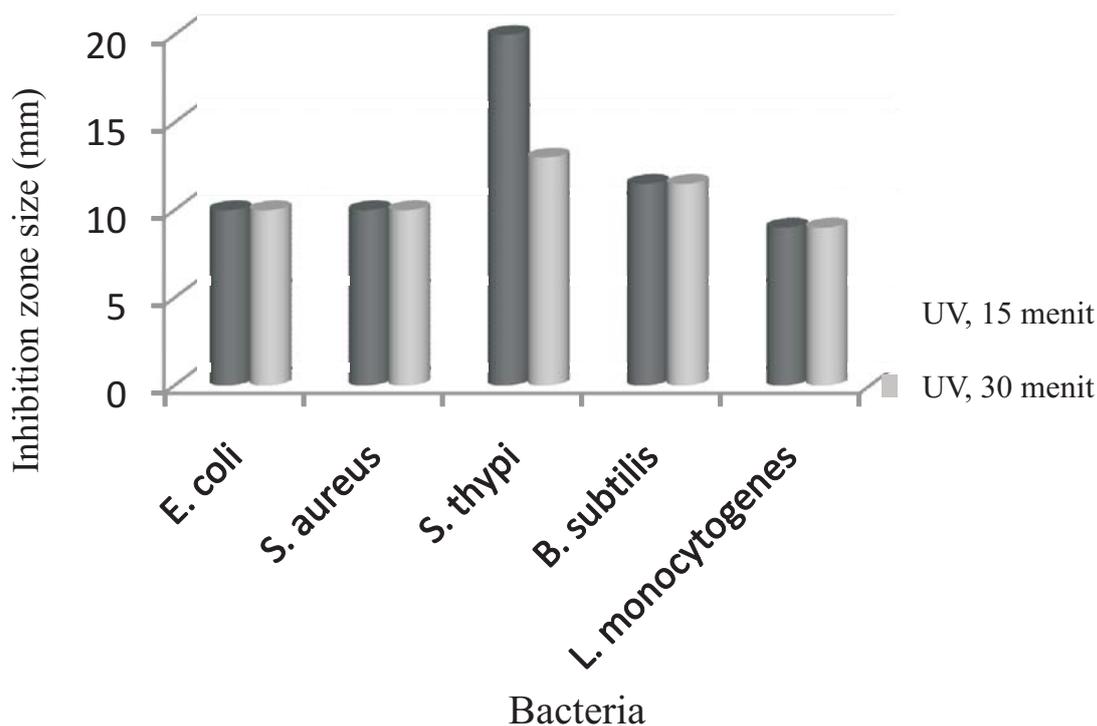


Fig 4 Effect of uv light on activity of antimicrobial compound from *Bacillus cereus* SS28, determined based on the size of the inhibition zone (mm).

Transmission Electron Microscopy. The effect of antimicrobial compound from *B. cereus* SS28 on bacterial cells was studied using *S. aureus* as a representative of Gram-positive cells. Morphological investigations were performed using 48-h *S. aureus* culture treated with antimicrobial compound from *B. cereus* SS28 (20 µg/ml). Control has exhibited typical coccus morphology of *S. aureus* (Fig 6). Untreated

S. aureus cells) shows a typically structured nucleus of *S. aureus* and a perfect cell wall (Fig 6A). After 48 hours of exposure to the antimicrobial compound, a slight alteration can be observed in the cell cytoplasm (Fig 6B), the cells exhibited notable alteration in cell cytoplasm. Bacterial cells completely collapsed 48 h after treatment with the antimicrobial compound (Fig 6).

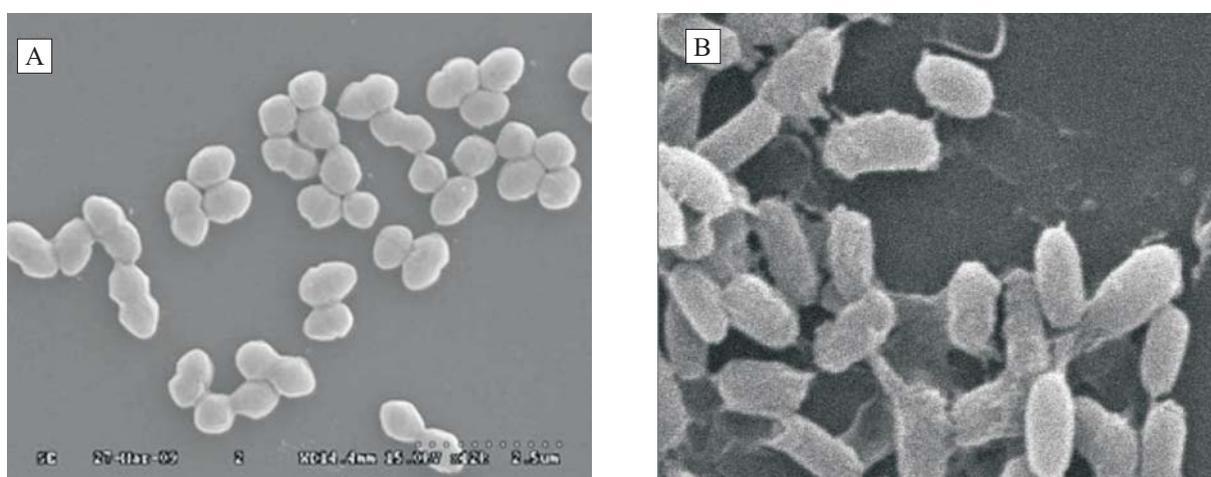


Fig 5 Scanning electron microscopy of cells *Staphylococcus aureus* control (a) (Bajpal et al. 2009) and after treatment with antimicrobial compound of *Bacillus cereus* SS28 (b).

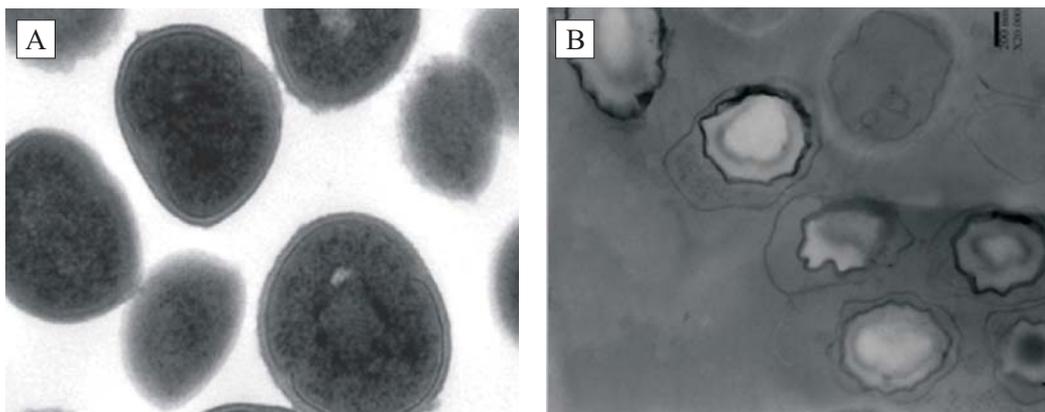


Fig 6 Transmission electron microscopy of cells *Staphylococcus aureus* control (A) (Santhana *et al.*, (2007) and after treatment with antimicrobial compound of *Bacillus cereus* SS28 (B).

DISCUSSION

Bacteriocin activity remained stable up to 24 h fermentation, then the activity started to drop after 28 h fermentation. Koroleva (1991) stated that most of the metabolism products resulted in the log phase were in the form of lactic acids, which causes the decrease in pH of the medium. This acidic condition will eventually inhibit growth of the respective bacteria (negative feedback effect).

Bacteriocin is extracellular secondary metabolite. The increase in the amount of biomass produced in the bacterial culture caused the increase in the amount of the bacteriocin produced. After reaching the stationary phase, the amount started to decrease (Boe 1996). Synthesis of bacteriocin by LAB occurred during the exponential growth phase, usually following the protein synthesis (Schnell *et al.*, 1998). Torkar and Matijasic (2003) who did research on the characterization of bacteriocins produced by *B. cereus* from milk and other dairy products, found that the production of bacteriocins entered the stationary phase after 10-16 hours of incubation. The research by Naclerio *et al.* (1993) on the production and activity of bacteriocins cerein from *B. cereus* present in the stationary phase also demonstrated similar result.

The highest antibacterial activity was exhibited at pH range 2 to 3, while inactivation occurred between pH 9 to 11. Khalil *et al.* (2009) showed that bacteriocins produced by *B. megaterium* 22 has activity antimicrobial against *S. thypimurium* at pH range 2-8. Naclerio *et al.* (1993) who studied the antimicrobial activity of bacteriocins cerein from *B. cereus*, found that the compound's activity was

stable between pH 3-12. Growth temperature plays an important role and is often correlated with bacteriocin production (Todorov and Dicks 2006).

Similar to the results of Alam *et al.* (2011), who stated that bacteriocin of *B. subtilis* BS15 retained activity up to 80 °C for 30 min, other bacteriocin produced by *L. lactis* ssp. diacetylactis was reported to maintain its activity even after boiling for up to 60 min. On the other hand, Lactacin F was reported to completely lose the activity when treated at 50 °C for 30 min (Kojic *et al.* 1991; Kim *et al.* 2005). Cleveland *et al.* (2001) suggested several potential advantages of bacteriocins to serve as biopreservatives, namely: a) the material is not toxic and susceptible to degradation by proteolytic enzymes because it is a protein compound, b) the material does not harm the intestinal microflora because it is easily digested by gastrointestinal enzymes, c) the material can reduce the chemicals as a food preservative, d) flexibility of use, and e) stability towards sufficiently broad range of pH and temperature that it is resistant to treatment processes involving acids and bases, as well as hot and cold conditions.

Antimicrobial activity of *B. cereus* SS28 was the highest against *S. thypi*, with inhibition zone diameter 20 mm, after 15 minutes exposure, which decreased to 10 mm after 30 minutes. *S. species* and other gram negative bacteria were sensitive to nisin and other bacteriocins after exposure to treatments that change the permeability barrier properties of the outer membrane (Stevens *et al.* 1991). Khalil *et al.* (2009) *B. megaterium* 19 bacteriocin was stable after 15 min exposure to UV light and was completely destroyed after 90 min.

The effect of antimicrobial compound from supernatant *B. cereus* SS28 from wall and cell membrane was investigated. It could be associated with the damage in the cell wall and cell membrane and subsequent lysis and reduction. Immediately after treatment, 80% of the *S. aureus* cell's surface appeared rough, which is quite different from the normal cells. In a previous study with *Layconostoc mesenteroides*, which has an inducible autolytic enzyme, bacteriocin treatment, pressurization or their combination did not only produce cell death and cell lysis, but also triggered the autolytic enzyme, which, by hydrolyzing the wall, disintegrated the cells (Bhunia *et al.* 1987; Kalchayanand *et al.* 2002).

Electron microscopy showed cell lysis after treatment with antimicrobial compound of *B. cereus* SS28. The cell damage caused by antimicrobial compound resembles that observed with a crude bacteriocin treatment (Ocana *et al.* 1999). Bizani *et al.* (2005) tried to investigate the effect of cerein 8A against *Bacillus cereus* spore. An approximately 4-5 log₁₀ reduction was observed when spores were plated in PCA containing 800 AU ml⁻¹. As cerein 8A concentration increased to 1600 AU ml⁻¹, complete inhibition of colony development was observed. When spores were treated with cerein 8A in BHI broth before plating, similar results were observed. The bactericidal effect of the antimicrobial compound from *Bacillus cereus* SS28 apparently works by disrupting the membrane function of target organisms.

To conclude antimicrobial bacteriocin from *B. cereus* SS28 was stable over a broad range of pH (between pH 2 to 11) and to heat-treatment at 121°C for 15 min. The antimicrobial activity was the highest at being heated at 70°C for 45 min and for 15 min of exposure to UV light. The main changes observed under SEM and TEM analyses were structural disorganization of the *S. aureus* cellular membrane 48 h after exposure to the antimicrobial compound of *B. cereus* SS28.

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